Nrf2-mediated neuroprotection response to recurrent hypoglycemia is insufficient to prevent cognitive impairment in a rodent model of type 1 diabetes

McNeilly, Alison D.; Gallagher, Jennifer R.; Dinkova-Kostova, Albena T.; Hayes, John D.; Sharkey, John; Ashford, Michael L. J.

Published in:
Diabetes

DOI:
10.2337/DB15-1653

Publication date:
2016

Document Version
Peer reviewed version

Nrf2-mediated neuroprotective response to recurrent hypoglycemia is insufficient to prevent cognitive impairment in a rodent model of type 1 diabetes

Journal:	*Diabetes*
Manuscript ID	DB15-1653.R1
Manuscript Type:	Original Article
Date Submitted by the Author:	n/a
Complete List of Authors:	McNeilly, Alison; University of Dundee, School of Medicine Gallagher, Jennifer; University of Dundee, School of Medicine Dinkova-Kostova, Albena; University of Dundee, School of Medicine Hayes, John; University of Dundee, School of Medicine Sharkey, John; University of Dundee, School of Medicine Ashford, Michael; University of Dundee, School of Medicine McCrimmon, Rory; University of Dundee, School of Medicine

Nrf2-mediated neuroprotection against recurrent hypoglycemia is insufficient to prevent cognitive impairment in a rodent model of type 1 diabetes

Running title: Nrf2 and cognitive impairment in T1D

Alison D. McNeilly1, Jennifer R. Gallagher1, Albena T. Dinkova-Kostova2, John D. Hayes2, John Sharkey1, 3, Michael L.J. Ashford1, and Rory J. McCrimmon1

1Division of Molecular and Clinical Medicine, School of Medicine, Ninewells Hospital and Medical School, Dundee DD1 9SY

2 Division of Cancer Research, School of Medicine, Ninewells Hospital and Medical School, Dundee DD1 9SY

3 Division of Neuroscience, School of Medicine, Ninewells Hospital and Medical School, Dundee DD1 9SY

Corresponding author:

Professor Rory J McCrimmon

Division of Molecular and Clinical Medicine,School of Medicine, University of Dundee, Dundee DD1 9SY

Email: r.mccrimmon@dundee.ac.uk

Telephone: +44 (0)1382383444

Fax: +44 (0)1382 383598

Manuscript Word Count: 4000

Figures: 3

Tables: 2

Supplementary Figure: 1

Supplementary Tables: 2

Abstract

It remains uncertain whether recurrent non-severe hypoglycemia (Hypo) results in long-term cognitive impairment in type 1 diabetes (T1D). Both T1D and Hypo can compromise host defenses against oxidative stress. This study tested the hypothesis that specifically in the T1D state, Hypo leads to cognitive impairment via a pathological response to oxidative stress. Wild-type (Control) and Nuclear factor-erythroid 2 p45-related factor 2 (Nrf2) null mice were studied. Eight groups of mice (Control and *Nrf2*$^{-/-}$ ±T1D and ±Hypo) were subject to recurrent, twice-weekly, insulin or saline injections over 4 weeks, after which cognitive function was assessed and brain tissue analyzed. Recurrent moderate hypoglycemia in T1D, but not Control mice significantly impaired cognitive performance, and this was associated with hippocampal oxidative damage and inflammation despite an enhanced expression of Nrf2 and its target genes *Hmox1* and *Nqo1*. In *Nrf2*$^{-/-}$mice, both T1D and Hypo independently resulted in impaired cognitive performance and this was associated with oxidative cell damage and marked inflammation. Together, these data suggest that Hypo induces an Nrf2-dependent antioxidant response in the hippocampus, which counteracts oxidative damage. However, in T1D this neuroprotective mechanism is insufficient to prevent neuronal oxidative damage, resulting in chronic deficits in working and long-term memory.

Abstract Word count: 197

Hypoglycemia is a common adverse side effect of insulin therapy in Type 1 Diabetes (T1D), largely due to hyperinsulinemia, a diminished counterregulatory response, and impaired awareness of hypoglycemia (1). The brain is especially vulnerable to hypoglycemia due to its high metabolic demand and minimal fuel stores. As such, there is potential for recurrent hypoglycemia to produce long-term neuronal damage. This is a source of major concern and fear for individuals with T1D (2), especially as studies in animals and humans have yielded inconsistent findings.

Profound hypoglycemia, sufficient to cause coma, results in brain damage in humans [e.g. (3; 4)], but epidemiological studies yield conflicting data on the impact of reversible severe and non-severe hypoglycemia on long-term cognitive function (5). For instance, 18 years follow-up of T1D individuals in the Diabetes Control and Complications Trial (DCCT) found no evidence of an association between severe hypoglycemia and cognitive decline (6). In contrast, prospective studies in pre-pubertal children with T1D, have reported that severe hypoglycemia may result in long-term neurologic damage and psychomotor retardation (7-11). A limitation of these studies is that the cognitive decline resulting from recurrent hypoglycemia may take place over many decades and accurate documentation of the frequency of hypoglycemia over such time scales is extremely difficult.

Studies in animal models offer the opportunity to address many of these questions over shorter time frames as well as to examine underlying mechanisms. This literature though is equally conflicting. Again, profound hypoglycemia (sufficient to induce an isoelectric EEG and/or multiple seizures) in animal models causes brain damage particularly in hippocampus and frontal cortex (12-15). However, significant neuronal damage under such conditions would be anticipated, and the relevance therefore to T1D where hypoglycemia of such severity is rare is not clear. In contrast, non-severe hypoglycemia does not appear to induce neuronal cell death (16) and long-term recurrent non-severe hypoglycemia has even been shown to protect against age-related cognitive decline (17). In addition, recurrent non-severe hypoglycemia in rodents potentially pre-conditions the brain protecting it to some extent from neurological damage resulting from subsequent very severe hypoglycemia (18).

An additional limitation of studies in animals is that many were primarily conducted using non-diabetic models. This is important, because in T1D, intermittent exposure to hypoglycemia will always occur in the context of chronic hyperglycemia of varying degrees. Chronic hyperglycemia (19), severe hypoglycemia (20; 21), and glucose recovery from hypoglycemia (13; 22), have each independently been shown to stimulate reactive oxygen species (ROS) production. In addition, chronic hyperglycemia may impair antioxidant defense mechanisms (23-25). Therefore, the ability of neurons to respond to non-severe hypoglycemia may be uniquely impaired in T1D increasing vulnerability of the brain particularly to oxidative stress, but this question has to date not been addressed.

To test this hypothesis, we studied insulin-treated T1D and non-diabetic rodent models that were exposed to intermittent episodes of non-severe hypoglycemia over 4 weeks and

examined the impact of these interventions on cognitive function and markers of oxidative stress and inflammation. Having demonstrated that recurrent hypoglycemia in T1D but not non-diabetic rodents induced defects in cognitive function that were associated with hippocampal inflammation and oxidative damage, we subsequently sought to clarify the role of the oxidative stress response by studying mice lacking the transcription factor Nuclear factor-erythroid 2 p45-related factor 2 (Nrf2) since it plays a critical role in regulating basal cellular antioxidant defenses as well as orchestrating responses to oxidative stress [for review see (26)]. Here we report that *Nrf2*$^{-/-}$ mice are very vulnerable to both T1D and recurrent hypoglycemia and that loss of Nrf2 resulted in strongly enhanced hippocampal inflammatory and oxidative damage responses to these two metabolic stimuli.

RESEARCH DESIGN AND METHODS

Experimental animals

Sixty-four adult male C57BL/6J mice (20-25g; Charles River, UK) and 30 male *Nrf2*$^{-/-}$ (20-25g) were used. Generation and genotyping of *Nrf2*$^{-/-}$ mice (kindly provided by Ken Itoh and Masayuki Yamamoto) have been described previously (27). Animals were fed *ad libitum*, on a 12:12h light:dark schedule. All animal procedures were approved by the University of Dundee Ethical Review Process and performed in accordance with UK Home Office regulations [under the auspices of Project Licence PIL60/4120].

Experimental groups

Groups of animals were randomly assigned to receive streptozotocin (STZ; 125mg/kg intraperitoneally; i.p.) to induce T1D or control (citrate acid buffer i.p.). Tail vein blood glucose (Accuread®) was measured 3 and 7 days post-STZ and a reading ≥16.0 mmol/l (288 mg/dl) regarded as diabetic. Animals failing to reach this were given a second injection of STZ and re-tested as above. Mice were subsequently sub-divided into recurrent hypoglycemia (Hypo) or control giving the following 4 test groups: 1) Control (Con), 2) Control + Hypo, 3) T1D, 4) T1D + Hypo (n=8 per group; see Supplementary Figure S1A). Similarly, for studies of *Nrf2*-null mice, animals were randomly allocated to the following 4 test groups: 1) *Nrf2*$^{-/-}$, 2) *Nrf2*$^{-/-}$ + Hypo, 3) T1D *Nrf2*$^{-/-}$, 4) T1D *Nrf2*$^{-/-}$ + Hypo (n=7-8 per group).

Surgery

Animals were anesthetized by isoflurane and Linbit® insulin implants inserted subcutaneously. Insulin replacement was used in an attempt to replicate more closely human insulin-treated T1D, and to ensure the animals remained healthy and showed positive weight trajectory over the 12 weeks of the experiment (See Supplemental Figures, S1B and C). However, for the purposes of this study where the interaction between chronic hyperglycemia and Hypo was being explored, insulin implants at half of the recommended dose (~0.05U/kg/day) were used (Figure 1A and C). Control animals were also anesthetized and subjected to sham surgery.

Recurrent hypoglycemia

Mice were subjected to 8 episodes of hypoglycemia (2 per week for 4 weeks; Figure S1A). Following a 4hr fast, basal glucose was measured from the tail vein and insulin was injected (0.75mU/g *Nrf2*$^{-/-}$, 1mU/g Control and WT animals and 4mU/g T1D and T1D *Nrf2*$^{-/-}$i.p.) to induce moderate hypoglycemia (2.5 – 3 mmol/l; Figure 1B and 1D). Hypoglycemia was maintained for 2hrs and animals allowed to return to euglycemic levels with food. Animals were monitored continuously during the hypoglycemic period. After 2 weeks, insulin doses were reduced to 0.5mU/g *Nrf2*$^{-/-}$, 0.75mU/g Control and WT animals and 2.5 - 3mU/g for T1D and T1D *Nrf2*$^{-/-}$ animals respectively. Any animal showing signs of physical impairment received glucose (1mg/kg i.p.). 2 animals in the Control group, 1 animal in the T1D group and 1 animal from the *Nrf2*$^{-/-}$ group required recovery on 1 occasion. Control animals were fasted and given saline injections. No animals suffered from seizures.

Behavioral Procedures

Cognition was assessed by novel object recognition (NOR) and spontaneous alternation tests (28; 29), at least 3 days after the last hypoglycemic episode (Figure S1A). Beckman Activity Box and Open field maze tasks were also made to exclude any confounding effects of altered activity or anxiety.

Novel object recognition

A simple hippocampal-mediated task based on the innate tendency of rodents to seek novelty was used (28). The primary outcome measure was the discrimination index (D3), calculated as the total time spent exploring the familiar objects/ total time spent exploring the novel object, for short-term (10 min) and long-term (24hr) memory.

Spontaneous alternation

This was used to test spatial working memory as described previously (29). Memory was assessed as a percent 4/5 alternation with an alternation counted when all four arms are visited within a span of five arm entries (29).

Activity box

Locomotor activity was assessed using a Beckman Activity Box. Animals, habituated to the box for 4 days, were placed into the box and allowed to explore freely for 15 minutes. Mobile, active and static counts were recorded.

Open field maze

Activity was recorded on film and later analysed by two independent scorers for total time spent within the inner and outer zones of the maze. Activity is presented as % total time spent in each zone.

Biochemical analyses

On completion of behavioral testing, animals were killed humanely, brain tissue dissected and flash frozen in liquid nitrogen for subsequent biochemical analyses.

Lipid peroxidation

The concentration of malondialdehyde (MDA) was determined in hippocampus using the thiobarbituric acid reactive substances (TBARS) assay as described in (30), and adapted for a 96-well plate format. The amount of MDA in the samples was determined spectrophotometrically at 532 nm and concentration determined from a standard curve. All samples were assayed in duplicate.

Protein carbonylation

Levels of carbonylated protein within the hippocampus were measured by ELISA (Caymen Chemicals Ltd). Protein carbonyl concentration was calculated using the following equation: Protein carbonyl (nmol/ml)=[(CA)/(*0.011 mM-1)](500 ml/200 ml), where CA is equal to the corrected absorbance (average absorbance of controls – average absorbance of samples).

Pro-inflammatory protein array

The total protein levels of a panel of inflammatory cytokines (IFNγ, IL-10, IL-12p70, IL-1β IL-2, IL-4, IL-5, IL-6, KC/GRO and TNFα were measured using the V-Plex Pro-inflammatory Panel (mouse) kit (MSD). Hippocampal homogenates (50µg) were assayed in duplicate and values presented relative to their appropriate control group (Con or *Nrf2*$^{-/-}$ respectively).

RNA extraction and PCR

Total RNA was extracted from hippocampal tissue using TRIzol® reagent (Invitrogen). Reverse transcription was performed with 1 ng RNA using SuperScript® III First Strand Synthesis system for RT (Invitrogen). Real-time PCR was performed using Taqman gene expression assays for the following genes: Nrf2 and Nrf2 target-genes (*Hmox1, Nqo1, Gsta1, Txn1, Txrnd1* and *Srxn1*(31)), the inflammatory genes Il-1β, Il-6, Tnfα, and Nos2, and house-keeping genes (Applied Biosystems; See Supplementary Table 1 for full details of primer/probe sequences). All samples were performed in triplicate and normalized to Actin. Values are expressed as a fold-change relative to Control.

Statistical analysis

Data were analyzed using SPSS version 18. Multivariate analysis of variance (ANOVA) was used to compare groups with treatment (TID) or genotype (*Nrf2*$^{-/-}$) and Hypo as between subject variables. Post-hoc analysis was performed using Tukey's multiple comparisons test. Data are expressed as mean values ±SEM. Statistical significance was set at $p<0.05$.

RESULTS

Recurrent hypoglycemia in wild-type and *Nrf2*$^{-/-}$ mice with or without T1D

As expected, blood glucose levels were higher in wild-type T1D compared to control animals over the 12-week study (Figure 1A; T1D x week $F(3.74,93.48)=35.89$; $p<0.05$). However, the level of hypoglycemia achieved was comparable between T1D and Control groups, (Figure

1B; Average glucose, Control=2.9±0.3 vs. TID=3.0±0.4 mmol/l; t=0.088; p=0.97) and did not change over the duration of the experiment (T1D F(1,26)=0.621; p=0.45).

Similarly, T1D *Nrf2*$^{-/-}$ mice had significantly higher fasting glucose levels when compared to non-diabetic *Nrf2*$^{-/-}$ animals for the duration of the study (Figure 1C ; T1D x week F(3.66,36.66)= 21.53; <0.01) but the degree of hypoglycemia achieved between T1D *Nrf2*$^{-/-}$ and *Nrf2*$^{-/-}$ animals was comparable (Figure 1D. Average glucose, *Nrf2*$^{-/-}$ =2.4±0.3 vs. TID *Nrf2*$^{-/-}$=3.1±0.6 mmol/l; t=0.062, p=0.95).

Recurrent hypoglycemia impairs cognitive function in T1D wild-type and *Nrf2*$^{-/-}$mice

The most significant cognitive defect found was in long-term memory as assessed by the NOR task in T1D + Hypo mice (Figure 2A; T1D x Hypo; F(1,30)=5.936, p<0.05). A less marked cognitive defect at this time point was also found in T1D mice (Figure 2A; T1D F(1,30)=52.40, p<0.05), but not in control or control + Hypo animals. These differences were not due to a reduction in time spent exploring the objects (effect of T1D and Hypo both p=ns). Performance on the NOR task after a 10min interval was intact in all groups (main effects T1D and Hypo on D3 indices and Exploration time; all p=ns), indicating that both the ability to learn the task and short-term memory for objects were intact

On the spontaneous alternation task, T1D + Hypo animals were also most affected, demonstrating a reduction in percentage alternations ([Total alternations/ total entries-4)*100] Figure 2B; T1D x Hypo (1,15)= 5.71; p<0.01) and fewer total alternations (T1D x HypoF(1,15)=7.734; p<0.01; Average total alternations; Control 46.29±3.66, Control + Hypo 48.36 ±3.63, T1D 47.10±4.67, T1D + Hypo 41.67±3.80). In contrast, control animals receiving Hypo showed a trend towards enhanced % alternation (Figure 2B; F(1,15)=3.04; p=0.10). There was no effect of T1D alone on the total number of entries (F(1,30)=2.92; p=ns).

As with wild-types, all *Nrf2*$^{-/-}$animals performed the short term NOR task above chance levels (D3 index of ≥0.2; F<1, p=ns), and as before there were no differences between groups in discrimination indices and time spent exploring the objects ((D3 indices and Exploration time; all F<1, p=ns). However, after 24 hours, only control *Nrf2*$^{-/-}$ animals were able to perform the task with both Hypo, T1D and Hypo+T1D markedly impairing long-term memory (Figure 2C; Hypo F(1,25)=12.09, p<0.01; T1D F(1,25)=6.95, p<0.05); T1D x Hypo; F(1,25)=6.24, p<0.05). These differences were not due to a reduction in time spent exploring the objects (Exploration time; all F<1, p=ns).

In both non-diabetic and T1D *Nrf2*$^{-/-}$ animals Hypo resulted in impaired working memory ([Total alternations/ total entries-4)*100] Figure 2D; Hypo F(1,25)=18.18; p<0.01; F<1 for the effect of T1D and T1D x Hypo). This impairment was accompanied by a significant reduction in the total number of alternations (Hypo F(1,25)=9.22, p<0.01; T1D F(1,25)=17.28, p<0.01; F<1, p=ns for effect of T1D x Hypo; Average total alternations; *Nrf2*$^{-/-}$ 28.35±2.78, *Nrf2*$^{-/-}$+ Hypo 21.00±2.80, T1D *Nrf2*$^{-/-}$24.86±4.11, T1D *Nrf2*$^{-/-}$ + Hypo 10.14±1.83). As with wild-type mice, there was no difference in total number of entries (T1D F(1,25)=4.12 p=0.776; F<1, p=ns for effect of Hypo and T1D x Hypo).

Assessments of locomotor activity and anxiety on the open field maze (data not shown), did not differ between any groups [active, mobile, or static counts (all F<1); time spent in central inner zone or the outer peripheral zone (all F<1)]

Recurrent hypoglycemia and chronic hyperglycemia act synergistically to activate an oxidative stress response

To explore the mechanisms underpinning the cognitive impairments demonstrated in working and long-term memory, transcript abundance of transcription factor Nrf2 as well as that of its target genes *Hmox1, Nqo1, Gsta1, Txn1, Txnrd1* and *Srxn1*were measured in the hippocampus of each animal model. Significant changes in gene expression were seen in response to T1D and Hypo in both wild type and *Nrf2*$^{-/-}$ animals (Table 1). Both T1D and T1D+Hypo increased mRNA for *Hmox1* and *Nqo1* in all animals, while a marked increase in Nrf2 expression was seen in the hippocampus of T1D+Hypo wild type mice. As expected, the expression of other Nrf2-regulated genes (*Gsta1, Txn1, Txnrd1* and *Srxn1*) appeared to be significantly diminished in all *Nrf2*$^{-/-}$mice irrespective of the metabolic model applied. Interestingly, mRNA for *Gsta1* was significantly decreased in response to Hypo in T1D and non-diabetic WT mice, and this effect was lost in *Nrf2*$^{-/-}$mice (Table 1).

Recurrent hypoglycemia and chronic hyperglycemia act synergistically to provoke ROS-induced cell damage in the hippocampus

To examine for evidence of ROS induced cellular damage, levels of lipid peroxidation and protein carbonylation were determined in hippocampal homogenates. Hypo in both non-diabetic and T1D mice increased lipid peroxidation within the hippocampus (Figure 3A; Hypo $F(1,30)=8.36$, $p<0.05$). Moreover, there was a T1D x Hypo interaction, indicating an additional stimulus to lipid peroxidation in T1D mice exposed to repeated hypoglycemia (T1D x Hypo $F(1,28)=6.24$ $p<0.05$). Protein carbonylation, an irreversible process resulting from exposure to ROS and an indicator of severe oxidative damage (32), was significantly elevated in the hippocampus only in T1D mice exposed to Hypo (Figure 3B; T1D x Hypo $F(1,28)=4.35$; $p<0.05$).

In *Nrf2*$^{-/-}$mice, hippocampal lipid peroxidation (Figure 3C) was increased by Hypo ($F(1,25)=76.86$; $p<0.01$), and greatest in T1D *Nrf2*$^{-/-}$ animals following Hypo (T1D x Hypo $F(1,25)=5.07$; $p<0.05$). Protein carbonylation was increased with Hypo (Figure 3D; Hypo $F(1,25)=38.08$; $p<0.01$), but not T1D ($F<1$, p=ns for T1D and T1D x Hypo).

Recurrent hypoglycemia and chronic hyperglycemia act synergistically to induce an inflammatory response within the hippocampus

To determine whether Hypo in T1D elicited an inflammatory response, cytokine protein levels were measured within hippocampal homogenates. In wild-type mice, IL-1β, IL-2, IL-4, IL-6 and TNFα were significantly enhanced in T1D animals whereas exposure to Hypo significantly increased levels of IL-12p70 and IL-5 (Table 2; all, $p<0.05$). The combination of Hypo + T1D resulted in a marked stimulus to the production of IL-1β, IL-2, TNFα, IL-4, IL-6, IL12p70, IL-5 (Table 2; all $p<.01$)

A similar, but more marked pattern in cytokine release was seen in *Nrf2*$^{-/-}$ animals. IL-1β, IL-2, TNFα, IL-4, IL-6, IL12p70, and IL-5 levels were increased in T1D, whereas IL-6, IL-12p70 and IL-5 were significantly increased following Hypo (Table 2; all $p< 0.05$). Again, in *Nrf2*$^{-/-}$mice the combination of Hypo + T1D resulted in a marked stimulus to the production of IL-1β, IL-2, TNFα, IL-4, IL-6, IL12p70, IL-5 (Table 2; all $p<0.001$). Interestingly, levels of all cytokines measured were increased even in untreated *Nrf2*$^{-/-}$mice, and amplified following each metabolic stimulus, although the pattern of change was nearly identical to that seen in wild-type mice.

The transcript abundance of the inflammatory genes *IL-1β*, *IL-6*, *Tnfα*, and *Nos2* were also checked and showed a similar pattern of change to protein levels of each cytokine, although these increases only reached significance in T1D and T1D+Hypo *Nrf2*$^{-/-}$ animals (Supplementary Table 2).

DISCUSSION

In this manuscript we show for the first time in a healthy insulin-treated animal model of T1D that recurrent non-severe hypoglycemia may impact on neuronal integrity and function. We show that 8 episodes of non-severe hypoglycemia in T1D over a 4-week period induces defects in memory consolidation and working memory, which is associated with persisting biochemical evidence of oxidative stress and inflammation in the hippocampus. Interestingly, protein synthesis, which is considered to play an integral role in memory consolidation, is disrupted at many levels by oxidative stress (33). In contrast and consistent with the work of others, recurrent hypoglycemia in non-diabetic rodents did not impair cognitive function (34). A key role for oxidative stress in inducing cognitive impairment was further illustrated by demonstrating a marked amplification of the hippocampal oxidative stress and inflammatory response in mice lacking the Nrf2 transcription factor (following recurrent hypoglycemia).

Pre-clinical and clinical studies suggest that marked glycemic variability maybe detrimental to humans (22; 35-37). In the current study, glucose variability is represented by three principal metabolic states, namely chronic hyperglycemia, acute hypoglycemia, and 'recovery' from acute hypoglycemia. Hypoglycemia can cause oxidative stress (20) and inflammation (21), and hypoglycemia-mediated ROS production can induce apoptosis (38; 39). However, the fact that no impairment in cognitive performance was seen following Hypo in wild-type mice implies that the intrinsic antioxidant capacity, coupled with the oxidative stress response orchestrated by Nrf2, is ordinarily sufficient to protect the neuron from the consequences of moderate glucose deprivation. Consistent with this, recurrent hypoglycemia despite recovery to normal glucose levels, resulted in cognitive impairment in *Nrf2*$^{-/-}$ mice. More recent studies in hippocampal slice preparations have shown that oxidative stress and neuronal death occur primarily in the recovery period from hypoglycemia during glucose reperfusion, and that the extent of oxidative stress correlates with the rise in glucose during recovery (13). In our model, glucose levels post-hypoglycemia were >16mmol/l and therefore glucose reperfusion into neurons that have experienced prolonged energy deprivation (as in ischemia reperfusion injury) may be the major contributor to oxidative stress. But Nrf2 protein levels and function are decreased in humans and rodents with diabetes (40; 41),

therefore the Nrf2-mediated defense mechanism may also be insufficient to prevent oxidative damage resulting from non-severe hypoglycemia in T1D. Neuronal damage secondary to severe hypoglycemia is exacerbated in T1D rats compared with non-diabetic controls (14). Taken together, our data provide robust evidence that recurrent non-severe hypoglycemia in T1D can provoke sufficient oxidative stress to induce a local inflammatory response and result in neuronal dysfunction, but cannot differentiate between potential effects of hypoglycemia *per se* or recovery to hyperglycemic levels. Future studies will be required to address this clinically important question.

Chronic hyperglycemia increases the production of ROS through mechanisms such as glucose auto-oxidation and non-enzymatic protein glycation (23), and lowers antioxidant defense mechanisms (24; 25) as well as serum free-radical trapping capacity (23). In the present study, both T1D wild-type and T1D *Nrf2*$^{-/-}$mice demonstrated impairments in memory consolidation. Neither model had tissue evidence of increased lipid peroxidation or protein carbonylation suggesting no significant oxidative damage, but both models had significantly increased levels of *Nrf2, Hmox-1, Nqo-1* and the inflammatory cytokines *IL-1β, IL-2, IL-4, IL-6* and *Tnfα*. Intriguingly, loss of Nrf2 amplified both the inflammatory response and cognitive defect. It is recognized that inflammatory responses are exacerbated in *Nrf2*$^{-/-}$ mice (42; 43), and such an outcome is consistent in humans and rodents with diabetes where Nrf2 levels and activity are diminished (40; 41). This is consistent with a recognized hierarchical response to oxidative stressors whereby modest levels of ROS activate an Nrf2-orchestrated adaptation whereas higher levels of ROS stimulate NF-κB and AP-1 to provide an additional defense mechanism (44). In this case, *Nrf2*$^{-/-}$mice would be anticipated to have higher levels of ROS under both hypo- and also hyperglycemia conditions leading to a pro-inflammatory state. Recent studies indicating a role for Nrf2 agonists in the treatment of diabetic nephropathy (43) and cardiomyopathy (45) that are associated with chronic hyperglycemia would be consistent with this possibility. The results of the present study would suggest that chronic hyperglycemia induces a pro-inflammatory condition through Nrf2-dependent and independent mechanisms.

A critical and novel finding in this paper is the important role of the transcription factor Nrf2 in initiating the oxidative stress response to hypoglycemia. Nrf2 is a transcription factor that dictates the intrinsic antioxidant capacity of cells under normal physiological conditions and also directs adaptation to oxidative stress. The activity of Nrf2 is itself regulated through a complex transcriptional/epigenetic and post-translational network in a manner that ensures its function increases during redox perturbation, inflammation and nutrient/energy fluxes, thereby enabling the factor to orchestrate adaptive responses to diverse forms of stress [for a review, see (26)]. Previously, Johnson and colleagues have reported that *Nrf2*$^{-/-}$ mice are significantly more sensitive to kainate neuronal toxicity than their wild-type counterparts, and used microarray analysis to show this was associated with markedly reduced expression in the hippocampus of *aldehyde oxidase 1, Gstm1, Gstm3, peroxiredoxin 1* (*Prdx1*) and *Prdx2* (46). In our study, the responses of *Nrf2*$^{+/+}$ and *Nrf2*$^{-/-}$ mice to T1D and Hypo were examined in separate experiments and we therefore cannot strictly compare gene expression profiles between wild-type and knockout mice. Nevertheless, it was apparent from our gene

expression analyses that large differences exist between the expression of *Gsta1, Gstm1* and *Srxn1*in the hippocampus of in *Nrf2*$^{+/+}$ and *Nrf2*$^{-/-}$ mice. In the future, it will be desirable to examine simultaneously the influence that loss of Nrf2 and genetic upregulation of Nrf2 (caused by diminished expression of Kelch-like ECH-associated protein 1 (Keap1), a negative regulator of Nrf2) has on gene expression and cognitive function following T1D and Hypo. It is notable that we found the levels of mRNA for Nrf2 were significantly upregulated by T1D, and very dramatically (by 12-fold) by T1D + Hypo. These results are consistent with a recent report that the protein levels of Nrf2 are increased in the diabetic wounds of humans and mice, and that pharmacological activation of Nrf2 promotes wound healing in T1D mice (47). Together with the heightened inflammation in T1D and T1D + Hypo*Nrf2*$^{-/-}$ mice relative to their wild-type counterparts, these findings suggest that a function of Nrf2 is to act as a "brake" to control inflammation. Our current results illustrate the critical importance of this function of Nrf2: indeed, inflammation is controlled and cognitive function is largely preserved in wild-type animals subjected to hypoglycemia, whereas inflammation is enhanced and cognitive function is severely impaired in their *Nrf2*$^{-/-}$ counterparts.

In conclusion, our study supports the hypothesis that recurrent moderate hypoglycemia in T1D may have long-term consequences on cognitive function. Our findings suggest that chronic hyperglycemia, recurrent hypoglycemia, and glucose reperfusion following hypoglycemia in T1D interact synergistically to induce pathological oxidative stress in vulnerable brain regions such as the hippocampus. Whether hypoglycemia *per se* or glucose recovery from hypoglycemia is the major contributor to oxidative damage in T1D cannot be determined from our studies although pre-clinical research suggests the recovery period is key. This has implications for clinical practice where treatment of hypoglycemia often leads to marked rebound hyperglycemia. Moreover, we provide evidence that the transcription factor Nrf2 may be integral to neuronal protection against oxidative stress during and following hypoglycemia, and in response to chronic hyperglycemia. This raises the possibility of targeting Nrf2 in developing therapies designed to prevent cellular damage in diabetes induced by recurrent hypoglycemia.

Acknowledgements

Funding: This work was supported by an award from the University of Dundee/ Wellcome Trust Translational Medical Research Fund (RJM), Diabetes UK (12/0004531) and Juvenile Diabetes Research Foundation (5-2011-464).

Author Contributions: A.D.M. designed and performed experiments and wrote manuscript. J.R.G. performed experiments. A.T.D-K. contributed to discussion and reviewed /edited manuscript. J.D.H. contributed to discussion and reviewed /edited manuscript. M.L.J.A. contributed to discussion and reviewed /edited manuscript. J.S. contributed to design of

experiments and discussion and research reviewed /edited manuscript. R.J.M designed experiments and wrote manuscript. R.J.M. is overall guarantor for the contents of the article.

REFERENCES

1. McCrimmon RJ, Sherwin RS: Hypoglycemia in type 1 diabetes. Diabetes 2010;59:2333-2339

2. McCrimmon RJ, Frier BM: Hypoglycaemia: the most feared complication of insulin therapy. Diabete & Metabolisme 1994;20:503-512

3. Chalmers J, Risk MT, Kean DM, Grant R, Ashworth B, Campbell IW: Severe amnesia after hypoglycemia. Clinical, psychometric, and magnetic resonance imaging correlations. Diabetes Care 1991;14:922-925

4. Fujioka M, Okuchi K, Hiramatsu KI, Sakaki T, Sakaguchi S, Ishii Y: Specific changes in human brain after hypoglycemic injury. Stroke; a journal of cerebral circulation 1997;28:584-587

5. McCrimmon RJ, Ryan CM, Frier BM: Diabetes and cognitive dysfunction. Lancet 2012;379:2291-2299

6. Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications Study Research G, Jacobson AM, Musen G, Ryan CM, Silvers N, Cleary P, Waberski B, Burwood A, Weinger K, Bayless M, Dahms W, Harth J: Long-term effect of diabetes and its treatment on cognitive function. New England Journal of Medicine 2007;356:1842-1852

7. Hershey T, Perantie DC, Warren SL, Zimmerman EC, Sadler M, White NH: Frequency and timing of severe hypoglycemia affects spatial memory in children with type 1 diabetes. Diabetes care 2005;28:2372-2377

8. Ryan CM, Atchison J, Puczynski S, Puczynski M, Arslanian S, Becker D: Mild hypoglycemia associated with deterioration of mental efficiency in children with insulin-dependent diabetes mellitus. The Journal of pediatrics 1990;117:32-38

9. Patino-Fernandez AM, Delamater AM, Applegate EB, Brady E, Eidson M, Nemery R, Gonzalez-Mendoza L, Richton S: Neurocognitive functioning in preschool-age children with type 1 diabetes mellitus. Pediatric diabetes 2010;11:424-430

10. Perantie DC, Wu J, Koller JM, Lim A, Warren SL, Black KJ, Sadler M, White NH, Hershey T: Regional brain volume differences associated with hyperglycemia and severe hypoglycemia in youth with type 1 diabetes. Diabetes care 2007;30:2331-2337

11. Perantie DC, Koller JM, Weaver PM, Lugar HM, Black KJ, White NH, Hershey T: Prospectively determined impact of type 1 diabetes on brain volume during development. Diabetes 2011;60:3006-3014

12. Auer RN, Wieloch T, Olsson Y, Siesjo BK: The distribution of hypoglycemic brain damage. Acta neuropathologica 1984;64:177-191

13. Suh SW, Gum ET, Hamby AM, Chan PH, Swanson RA: Hypoglycemic neuronal death is triggered by glucose reperfusion and activation of neuronal NADPH oxidase. J Clin Invest 2007;117:910-918

14. Bree AJ, Puente EC, Daphna-Iken D, Fisher SJ: Diabetes increases brain damage caused by severe hypoglycemia. American journal of physiology Endocrinology and metabolism 2009;297:E194-201

15. Won SJ, Yoo BH, Kauppinen TM, Choi BY, Kim JH, Jang BG, Lee MW, Sohn M, Liu J, Swanson RA, Suh SW: Recurrent/moderate hypoglycemia induces hippocampal dendritic injury, microglial activation, and cognitive impairment in diabetic rats. Journal of neuroinflammation 2012;9:182

16. Yamada KA, Rensing N, Izumi Y, De Erausquin GA, Gazit V, Dorsey DA, Herrera DG: Repetitive hypoglycemia in young rats impairs hippocampal long-term potentiation. Pediatr Res 2004;55:372-379

17. McNay EC, Williamson A, McCrimmon RJ, Sherwin RS: Cognitive and neural hippocampal effects of long-term moderate recurrent hypoglycemia. Diabetes 2006;55:1088-1095

18. Puente EC, Silverstein J, Bree AJ, Musikantow DR, Wozniak DF, Maloney S, Daphna-Iken D, Fisher SJ: Recurrent moderate hypoglycemia ameliorates brain damage and cognitive dysfunction induced by severe hypoglycemia. Diabetes 59:1055-1062

19. Butterfield DA, Di Domenico F, Barone E: Elevated risk of type 2 diabetes for development of Alzheimer disease: a key role for oxidative stress in brain. Biochim Biophys Acta 2014;1842:1693-1706

20. Singh P, Jain A, Kaur G: Impact of hypoglycemia and diabetes on CNS: correlation of mitochondrial oxidative stress with DNA damage. Molecular and cellular biochemistry 2004;260:153-159

21. Wright RJ, Newby DE, Stirling D, Ludlam CA, Macdonald IA, Frier BM: Effects of acute insulin-induced hypoglycemia on indices of inflammation: putative mechanism for aggravating vascular disease in diabetes. Diabetes care 2010;33:1591-1597

22. Ceriello A, Novials A, Ortega E, La Sala L, Pujadas G, Testa R, Bonfigli AR, Esposito K, Giugliano D: Evidence that hyperglycemia after recovery from hypoglycemia worsens endothelial function and increases oxidative stress and inflammation in healthy control subjects and subjects with type 1 diabetes. Diabetes 2012;61:2993-2997

23. Dominguez C, Ruiz E, Gussinye M, Carrascosa A: Oxidative stress at onset and in early stages of type 1 diabetes in children and adolescents. Diabetes care 1998;21:1736-1742

24. Marra G, Cotroneo P, Pitocco D, Manto A, Di Leo MA, Ruotolo V, Caputo S, Giardina B, Ghirlanda G, Santini SA: Early increase of oxidative stress and reduced antioxidant defenses in patients with uncomplicated type 1 diabetes: a case for gender difference. Diabetes care 2002;25:370-375

25. Vucic M, Gavella M, Bozikov V, Ashcroft SJ, Rocic B: Superoxide dismutase activity in lymphocytes and polymorphonuclear cells of diabetic patients. European journal of clinical chemistry and clinical biochemistry : journal of the Forum of European Clinical Chemistry Societies 1997;35:517-521

26. Hayes JD, Dinkova-Kostova AT: The Nrf2 regulatory network provides an interface between redox and intermediary metabolism. Trends Biochem Sci 2014;39:199-218

27. Chowdhry S, Nazmy MH, Meakin PJ, Dinkova-Kostova AT, Walsh SV, Tsujita T, Dillon JF, Ashford ML, Hayes JD: Loss of Nrf2 markedly exacerbates nonalcoholic steatohepatitis. Free radical biology & medicine 2010;48:357-371

28. Langston RF, Wood ER: Associative recognition and the hippocampus: differential effects of hippocampal lesions on object-place, object-context and object-place-context memory. Hippocampus 2010;20:1139-1153

29. McNay EC, Fries TM, Gold PE: Decreases in rat extracellular hippocampal glucose concentration associated with cognitive demand during a spatial task. Proceedings of the National Academy of Sciences of the United States of America 2000;97:2881-2885

30. Mihara M, Uchiyama M: Determination of malonaldehyde precursor in tissues by thiobarbituric acid test. Analytical biochemistry 1978;86:271-278

31. Gorrini C, Harris IS, Mak TW: Modulation of oxidative stress as an anticancer strategy. Nature reviews Drug discovery 2013;12:931-947

32. Jacob KD, Noren Hooten N, Trzeciak AR, Evans MK: Markers of oxidant stress that are clinically relevant in aging and age-related disease. Mechanisms of ageing and development 2013;134:139-157

33. Shenton D, Smirnova JB, Selley JN, Carroll K, Hubbard SJ, Pavitt GD, Ashe MP, Grant CM: Global translational responses to oxidative stress impact upon multiple levels of protein synthesis. J Biol Chem 2006;281:29011-29021

34. McNay EC, Teske JA, Kotz CM, Dunn-Meynell A, Levin BE, McCrimmon RJ, Sherwin RS: Long-term, intermittent, insulin-induced hypoglycemia produces marked obesity without hyperphagia or insulin resistance: a model for weight gain with intensive insulin therapy. Am J Physiol Endocrinol Metab 2013;304:E131-138

35. Rehni AK, Nautiyal N, Perez-Pinzon MA, Dave KR: Hyperglycemia / hypoglycemia-induced mitochondrial dysfunction and cerebral ischemic damage in diabetics. Metabolic brain disease 2014;

36. Brownlee M: The pathobiology of diabetic complications: a unifying mechanism. Diabetes 2005;54:1615-1625

37. Russo VC, Higgins S, Werther GA, Cameron FJ: Effects of fluctuating glucose levels on neuronal cells in vitro. Neurochemical research 2012;37:1768-1782

38. Manna SK, Zhang HJ, Yan T, Oberley LW, Aggarwal BB: Overexpression of manganese superoxide dismutase suppresses tumor necrosis factor-induced apoptosis and activation of nuclear transcription factor-kappaB and activated protein-1. The Journal of biological chemistry 1998;273:13245-13254

39. Nomura K, Imai H, Koumura T, Arai M, Nakagawa Y: Mitochondrial phospholipid hydroperoxide glutathione peroxidase suppresses apoptosis mediated by a mitochondrial death pathway. The Journal of biological chemistry 1999;274:29294-29302

40. Tan Y, Ichikawa T, Li J, Si Q, Yang H, Chen X, Goldblatt CS, Meyer CJ, Li X, Cai L, Cui T: Diabetic downregulation of Nrf2 activity via ERK contributes to oxidative stress-induced insulin resistance in cardiac cells in vitro and in vivo. Diabetes 2011;60:625-633

41. He HJ, Wang GY, Gao Y, Ling WH, Yu ZW, Jin TR: Curcumin attenuates Nrf2 signaling defect, oxidative stress in muscle and glucose intolerance in high fat diet-fed mice. World journal of diabetes 2012;3:94-104

42. Tebay LE, Robertson H, Durant ST, Vitale SR, Penning TM, Dinkova-Kostova AT, Hayes JD: Mechanisms of activation of the transcription factor Nrf2 by redox stressors, nutrient cues, and energy status and the pathways through which it attenuates degenerative disease. Free Radic Biol Med 2015;88:108-146

43. Jiang T, Huang Z, Lin Y, Zhang Z, Fang D, Zhang DD: The protective role of Nrf2 in streptozotocin-induced diabetic nephropathy. Diabetes 2010;59:850-860

44. Hamanaka RB, Chandel NS: Mitochondrial reactive oxygen species regulate cellular signaling and dictate biological outcomes. Trends Biochem Sci 2010;35:505-513

45. Wang Y, Sun W, Du B, Miao X, Bai Y, Xin Y, Tan Y, Cui W, Liu B, Cui T, Epstein PN, Fu Y, Cai L: Therapeutic effect of MG-132 on diabetic cardiomyopathy is associated with its suppression of proteasomal activities: roles of Nrf2 and NF-kappaB. Am J Physiol Heart Circ Physiol 2013;304:H567-578

46. Kraft AD, Lee JM, Johnson DA, Kan YW, Johnson JA: Neuronal sensitivity to kainic acid is dependent on the Nrf2-mediated actions of the antioxidant response element. Journal of neurochemistry 2006;98:1852-1865

47. Long M, Rojo de la Vega M, Wen Q, Bharara M, Jiang T, Zhang R, Zhou S, Wong PK, Wondrak GT, Zheng H, Zhang DD: An Essential Role of NRF2 in Diabetic Wound Healing. Diabetes 2016;65:780-793

Figure Legends

Figure 1. Physiological profile of diabetic and non-diabetic WT and *Nrf2*$^{-/-}$ mice exposed to recurrent hypoglycemia. A and C: Body weight was comparable between experimental groups and unaltered by either genotype or recurrent hypoglycemia. B and D: Example of typical insulin induced hypoglycemic event in WT and *Nrf2*$^{-/-}$ animals with a comparable level of hypoglycemia achieved despite *Nrf2*$^{-/-}$ animals receiving significantly lower insulin doses (starting dose 0.75mU/g WT control vs. 0.5mU/g *Nrf2*$^{-/-}$ animals). Control (Con), Con + Hypo, T1D and T1D + Hypo all n=8 per group. *Nrf2*$^{-/-}$, *Nrf2*$^{-/-}$+ Hypo, T1D *Nrf2*$^{-/-}$ and T1D *Nrf2*$^{-/-}$ + Hypo all n=7-8 per group. Results represent mean values ± SEM. Data were analyzed by 2-way ANOVA with T1D and Hypo as between subject factors followed by Tukey's post hoc test *p<0.05, **p<0.01 Control or *Nrf2*$^{-/-}$vs T1D or T1D *Nrf2*$^{-/-}$, ♯p<0.05, ♯♯p<0.01 Hypo vs. No Hypo.

Figure 2. Recurrent hypoglycemia (Hypo) in T1D mice impairs cognitive performance in novel-object recognition (NOR) and Spontaneous Alternation tasks. A: Discrimination index (D3) demonstrating that T1D mice are significantly impaired when tested in the 24hr NOR task. This is exacerbated in T1D animals following Hypo. B: Mean 4/5 alternation performance on a closed arm plus maze, expressed as a percentage of possible alternations is significantly reduced in T1D animals following Hypo. C: *Nrf2*$^{-/-}$ and T1D *Nrf2*$^{-/-}$ mice following Hypo were significantly impaired when tested in the 24hr NOR task, and D: Mean 4/5 alternation performance on a closed arm plus maze, expressed as a percentage of possible alternations is significantly reduced in *Nrf2*$^{-/-}$+ Hypo and T1D *Nrf2*$^{-/-}$+ Hypo animals when compared to their control (no hypo) counterparts. Control (Con), Con + Hypo, T1D and T1D + Hypo all n=8 per group. *Nrf2*$^{-/-}$, *Nrf2*$^{-/-}$+ Hypo, T1D *Nrf2*$^{-/-}$ and T1D *Nrf2*$^{-/-}$ + Hypo all n=7-8 per group. Results represent mean values ± SEM. Data were analyzed by 2-way ANOVA with T1D and Hypo as between subject factors followed by Tukey's post hoc test *p<0.05 Control or *Nrf2*$^{-/-}$vs T1D or T1D *Nrf2*$^{-/-}$, ♯p<0.05, ♯♯p<0.01 Hypo vs. No Hypo

Figure 3. Recurrent hypoglycemia in T1D is associated with increased markers of oxidative damage. The levels of lipid peroxidation were determined using the thiobarbituric acid reactive substances (TBARS) assay and protein carbonylation by ELISA. A: The levels of lipid peroxidation and B: protein carbonylation were significantly elevated following Hypo and further enhanced in T1D mice. The levels of C. Lipid peroxidation and D: protein carbonylation were augmented following Hypo in *Nrf2*$^{-/-}$ mice and enhanced further in T1D *Nrf2*$^{-/-}$ + Hypo animals. Control (Con), Con + Hypo, T1D and T1D + Hypo all n=8 per group. *Nrf2*$^{-/-}$, *Nrf2*$^{-/-}$+ Hypo, T1D *Nrf2*$^{-/-}$ and T1D *Nrf2*$^{-/-}$ + Hypo all n=7-8 per group. Results represent mean values ± SEM. Data were analyzed by 2-way ANOVA with T1D and Hypo as between subject factors followed by Tukey's post hoc test *p<0.05 Control or *Nrf2*$^{-/-}$vs T1D or T1D *Nrf2*$^{-/-}$, ♯p<0.05, ♯♯p<0.01 Hypo vs. No Hypo.

Tables 1. Recurrent hypoglycemia and T1D in both Wild-type and Nrf2$^{-/-}$ mice is associated with modulation of genes involved in mediating anti-oxidant and redox systems. mRNA was extracted and processed for real-time PCR to evaluate changes in gene expression of Hemeoxygenase 1 (Hmox1), Nrf2, NAD(P)H:quinoneoxidoreductase (Nqo1), Thioredoxinreductase 1 (Txnrd1), Sulfiredoxin 1 (Srxn1), Thioredoxin 1 (Txn1) and Glutathione*S*-transferase Alpha subunit 1 (Gsta1). Control (Con), Con + Hypo, T1D and T1D + Hypo all n=8 per group. *Nrf2*$^{-/-}$, *Nrf2*$^{-/-}$+ Hypo, T1D *Nrf2*$^{-/-}$ and T1D *Nrf2*$^{-/-}$ + Hypo all n=7-8 per group. Results represent mean values ± SEM. Data were analyzed within genotype by 2-way ANOVA with T1D and Hypo as between subject factors followed by Tukey's post hoc test *p<0.05, **p<0.01 Control or *Nrf2*$^{-/-}$vs T1D or T1D *Nrf2*$^{-/-}$, ♯p<0.05, ♯♯p<0.01 Hypo vs. No Hypo

Gene	Control	Control + Hypo	T1D	T1D + Hypo		Nrf2$^{-/-}$	Nrf2$^{-/-}$ + Hypo	T1D Nrf2$^{-/-}$	T1D Nrf2$^{-/-}$ + Hypo
Hmox-1	1.00±0.28	1.15±0.14	1.34±0.14*	1.88±0.55*$^{\sharp}$		0.71±0.07	0.49±0.08	1.05±0.11**	1.15±0.11**$^{\sharp}$
Nrf2	1.00±0.15	1.14±0.18	1.86±0.18*	12.13±0.10*$^{\sharp}$		n/d	n/d	n/d	n/d
Nqo1	1.00±0.35	1.53±0.17	2.11±0.28*	3.20±0.44*$^{\sharp}$		1.94±0.13	1.78±0.12	2.18±0.31*	2.74±0.24*$^{\sharp}$
Txnrd1	1.00±0.19	1.07±0.10	1.08±0.10	1.09±0.13		0.79±0.05	0.95±0.12	0.60±0.08	0.59±0.05
Srxn1	1.00±0.09	0.94±0.06	1.06±0.03	1.13±0.14		0.58±0.10	0.71±0.16	0.43±0.05	0.48±0.04
Txn1	1.00±0.16	0.96±0.11	1.28±0.19	1.32±0.23		0.82±0.06	0.74±0.11	0.73±0.11	0.76±0.11
Gsta1	1.00±0.13	0.50±0.76$^{\sharp}$	1.00±0.06	0.53±0.16$^{\sharp}$		0.11±0.04	0.22±0.07	0.10±0.04	0.10±0.04

Table 2. Recurrent hypoglycemia and T1D induce inflammation within the hippocampus in both WT T1D animals and *Nrf2*$^{-/-}$animals respectively. Levels of a number of common inflammatory cytokines were measured within hippocampal homogenates by ELISA. Con, Con + Hypo, T1D and T1D + Hypo all n=8 per group. *Nrf2*$^{-/-}$, *Nrf2*$^{-/-}$+ Hypo, T1D *Nrf2*$^{-/-}$ and T1D *Nrf2*$^{-/-}$+ Hypo all n=7-8 per group. Results represent mean values ± SEM. Data were analyzed within genotype by 2-way ANOVA with T1D and Hypo as between subject factors followed by Tukey's post hoc test * p<0.05, test *p<0.05, **p<0.01 Control or *Nrf2*$^{-/-}$vs T1D or T1D *Nrf2*$^{-/-}$, ♯p<0.05, ♯♯p<0.01 Hypo vs. No Hypo.

Protein (pg/mg)	**Control**	**Control + Hypo**	**T1D**	**T1D + Hypo**		**Nrf2$^{-/-}$**	**Nrf2$^{-/-}$ + Hypo**	**T1D Nrf2$^{-/-}$**	**T1D Nrf2$^{-/-}$ + Hypo**
IFNg	1.26±0.19	1.01±0.19	1.21±0.19	1.16±0.29		2.04 ±0.13	1.99±0.24	1.97±0.21	1.73±0.30
KC/GRO	57.60±7.42	68.86±10.73	65.15±9.51	59.57±7.03		99.49±7.24	95.35±12.47	102.7±12.12	116.7±21.47
IL-1b	1.63±0.14	1.72±0.26	2.68±0.25**	3.15±0.43**♯		2.96±0.49	2.62±0.39	6.95±0.80*	9.40±1.23*♯
IL-2	5.20±1.10	6.19±0.93	6.80±1.27*	10.72±1.23*♯		6.47±0.76	7.10±1.10	10.91±1.15**	10.88±0.86**♯
TNFa	1.87±0.45	1.39±0.73	4.28±0.44*	7.37±0.61**♯		4.97±0.86	7.34±1.40	10.41±2.28*	10.75±1.98*♯
IL-4	1.56±0.26	1.32±0.26	3.05±0.52**	4.35±0.34**♯		2.16±0.32	3.28±0.53	4.96±0.58**	5.34±0.57**♯
IL-6	85.54±13.29	106.4±16.11	146.8±17.30**	178.3±9.32**♯		99.48±11.59	140.4±3.99♯	145.4±8.58**	149.6±6.88**♯
IL-12p70	20.20±1.31	25.84±1.15♯	24.83±1.71	27.65±02.09*♯		20.00±3.25	27.63±2.83♯♯	22.02±3.84*	35.23±4.27*♯♯

IL-5	5.39±0.68	7.17±0.41$^{\#}$	5.11±0.69	8.48±0.74*$^{\#\#}$		5.48±0.66	6.78±0.31$^{\#}$	8.45±0.59**	7.58±0.22**$^{\#}$

.

Nrf2-mediated neuroprotection against recurrent hypoglycemia is insufficient to prevent cognitive impairment in a rodent model of type 1 diabetes

Running title: Nrf2 and cognitive impairment in T1D

Alison D. McNeilly1, Jennifer R. Gallagher1, Albena T. Dinkova-Kostova2, John D. Hayes2, John Sharkey1, 3, Michael L.J. Ashford1, and Rory J. McCrimmon1

1Division of Molecular and Clinical Medicine, School of Medicine, Ninewells Hospital and Medical School, Dundee DD1 9SY

2 Division of Cancer Research, School of Medicine, Ninewells Hospital and Medical School, Dundee DD1 9SY

3 Division of Neuroscience, School of Medicine, Ninewells Hospital and Medical School, Dundee DD1 9SY

Corresponding author:

Professor Rory J McCrimmon

Division of Molecular and Clinical Medicine,School of Medicine, University of Dundee, Dundee DD1 9SY

Email: r.mccrimmon@dundee.ac.uk

Telephone: +44 (0)1382383444

Fax: +44 (0)1382 383598

Manuscript Word Count: 4000

Figures: 3

Tables: 2

Supplementary Figure: 1

Supplementary Tables: 2

Abstract

It remains uncertain whether recurrent non-severe hypoglycemia (Hypo) results in long-term cognitive impairment in type 1 diabetes (T1D). Both T1D and Hypo can compromise host defenses against oxidative stress. This study tested the hypothesis that specifically in the T1D state, Hypo leads to cognitive impairment via a pathological response to oxidative stress. Wild-type (Control) and Nuclear factor-erythroid 2 p45-related factor 2 (Nrf2) null mice were studied. Eight groups of mice (Control and *Nrf2*$^{-/-}$ ±T1D and ±Hypo) were subject to recurrent, twice-weekly, insulin or saline injections over 4 weeks, after which cognitive function was assessed and brain tissue analyzed. Recurrent moderate hypoglycemia in T1D, but not Control mice significantly impaired cognitive performance, and this was associated with hippocampal oxidative damage and inflammation despite an enhanced expression of Nrf2 and its target genes *Hmox1* and *Nqo1*. In *Nrf2*$^{-/-}$mice, both T1D and Hypo independently resulted in impaired cognitive performance and this was associated with oxidative cell damage and marked inflammation. Together, these data suggest that Hypo induces an Nrf2-dependent antioxidant response in the hippocampus, which counteracts oxidative damage. However, in T1D this neuroprotective mechanism is insufficient to prevent neuronal oxidative damage, resulting in chronic deficits in working and long-term memory.

Abstract Word count: 197

Hypoglycemia is a common adverse side effect of insulin therapy in Type 1 Diabetes (T1D), largely due to hyperinsulinemia, a diminished counterregulatory response, and impaired awareness of hypoglycemia (1). The brain is especially vulnerable to hypoglycemia due to its high metabolic demand and minimal fuel stores. As such, there is potential for recurrent hypoglycemia to produce long-term neuronal damage. This is a source of major concern and fear for individuals with T1D (2), especially as studies in animals and humans have yielded inconsistent findings.

Profound hypoglycemia, sufficient to cause prolonged coma, results in brain damage in humans [e.g. (3; 4)], but epidemiological studies yield conflicting data on the impact of reversible severe and non-severe hypoglycemia on long-term cognitive function (5). For instance, 18 years follow-up of T1D individuals in the Diabetes Control and Complications Trial (DCCT) found no evidence of an association between severe hypoglycemia and cognitive decline (6). In contrast, prospective studies in pre-pubertal children with T1D, have reported that severe hypoglycemia may result in long-term neurologic damage and psychomotor retardation (7-11). A limitation of these studies is that the cognitive decline resulting from recurrent hypoglycemia may take place over many decades and accurate documentation of the frequency of hypoglycemia over such time scales is extremely difficult.

Studies in animal models offer the opportunity to address many of these questions over shorter time frames as well as to examine underlying mechanisms. This literature though is equally conflicting. Again, profound hypoglycemia (sufficient to induce an isoelectric EEG and/or multiple seizures) in animal models causes brain damage particularly in hippocampus and frontal cortex (12-15). However, significant neuronal damage under such conditions would be anticipated, and the relevance therefore to T1D where hypoglycemia of such severity is rare is not clear. In contrast, non-severe hypoglycemia does not appear to induce neuronal cell death (16) and long-term recurrent non-severe hypoglycemia has even been shown to protect against age-related cognitive decline (17). In addition, recurrent non-severe hypoglycemia in rodents potentially pre-conditions the brain protecting it to some extent from neurological damage resulting from subsequent very severe hypoglycemia (18).

An additional limitation of studies in animals is that many were primarily conducted using non-diabetic models. This is important, because in T1D, intermittent exposure to hypoglycemia will always occur in the context of chronic hyperglycemia of varying degrees. Chronic hyperglycemia (19), severe hypoglycemia (20; 21), and glucose recovery from hypoglycemia (13; 22), have each independently been shown to stimulate reactive oxygen species (ROS) production. In addition, chronic hyperglycemia may impair antioxidant defense mechanisms (23-25). Therefore, the ability of neurons to respond to non-severe hypoglycemia may be uniquely impaired in T1D increasing vulnerability of the brain particularly to oxidative stress, but this question has to date not been addressed.

To test this hypothesis, we studied insulin-treated T1D and non-diabetic rodent models that were exposed to intermittent episodes of non-severe hypoglycemia over 4 weeks and

examined the impact of these interventions on cognitive function and markers of oxidative stress and inflammation. Having demonstrated that recurrent hypoglycemia in T1D but not non-diabetic rodents induced defects in cognitive function that were associated with hippocampal inflammation and oxidative damage, we subsequently sought to clarify the role of the oxidative stress response by studying mice lacking the transcription factor Nuclear factor-erythroid 2 p45-related factor 2 (Nrf2) since it plays a critical role in regulating basal cellular antioxidant defenses as well as orchestrating responses to oxidative stress [for review see (26)]. Here we report that *Nrf2*$^{-/-}$ mice are very vulnerable to both T1D and recurrent hypoglycemia and that loss of Nrf2 resulted in strongly enhanced hippocampal inflammatory and oxidative damage responses to these two metabolic stimuli.

RESEARCH DESIGN AND METHODS

Experimental animals

Sixty-four adult male C57BL/6J mice (20-25g; Charles River, UK) and 30 male *Nrf2*$^{-/-}$ (20-25g) were used. Generation and genotyping of *Nrf2*$^{-/-}$ mice (kindly provided by Ken Itoh and Masayuki Yamamoto) have been described previously (27). Animals were fed *ad libitum*, on a 12:12h light:dark schedule. All animal procedures were approved by the University of Dundee Ethical Review Process and performed in accordance with UK Home Office regulations [under the auspices of Project Licence PIL60/4120].

Experimental groups

Groups of animals were randomly assigned to receive streptozotocin (STZ; 125mg/kg intraperitoneally; i.p.) to induce T1D or control (citrate acid buffer i.p.). Tail vein blood glucose (Accuread®) was measured 3 and 7 days post-STZ and a reading ≥16.0 mmol/l (288 mg/dl) regarded as diabetic. Animals failing to reach this were given a second injection of STZ and re-tested as above. Mice were subsequently sub-divided into recurrent hypoglycemia (Hypo) or control giving the following 4 test groups: 1) Control (Con), 2) Control + Hypo, 3) T1D, 4) T1D + Hypo (n=8 per group; see Supplementary Figure S1A). Similarly, for studies of *Nrf2*-null mice, animals were randomly allocated to the following 4 test groups: 1) *Nrf2*$^{-/-}$, 2) *Nrf2*$^{-/-}$ + Hypo, 3) T1D *Nrf2*$^{-/-}$, 4) T1D *Nrf2*$^{-/-}$ + Hypo (n=7-8 per group).

Surgery

Animals were anesthetized by isoflurane and Linbit® insulin implants inserted subcutaneously. Insulin replacement was used in an attempt to replicate more closely human insulin-treated T1D, and to ensure the animals remained healthy and showed positive weight trajectory over the 12 weeks of the experiment (See Supplemental Figures, S1B and C). However, for the purposes of this study where the interaction between chronic hyperglycemia and Hypo was being explored, insulin implants at half of the recommended dose (~0.05U/kg/day) were used (Figure 1A and C). Control animals were also anesthetized and subjected to sham surgery.

Recurrent hypoglycemia

Mice were subjected to 8 episodes of hypoglycemia (2 per week for 4 weeks; Figure S1A). Following a 4hr fast, basal glucose was measured from the tail vein and insulin was injected (0.75mU/g *Nrf2*$^{-/-}$, 1mU/g Control and WT animals and 4mU/g T1D and T1D *Nrf2*$^{-/-}$i.p.) to induce moderate hypoglycemia (2.5 – 3 mmol/l; Figure 1B and 1D). Hypoglycemia was maintained for 2hrs and animals allowed to return to euglycemic levels with food. Animals were monitored continuously during the hypoglycemic period. After 2 weeks, insulin doses were reduced to 0.5mU/g *Nrf2*$^{-/-}$, 0.75mU/g Control and WT animals and 2.5 - 3mU/g for T1D and T1D *Nrf2*$^{-/-}$ animals respectively. Any animal not eating or showing signs of locomotor/physical impairment received glucose (1mg/kg i.p.). In total 2 animals in the Control group, 1 animal in the T1D group and 1 animal from the *Nrf2*$^{-/-}$ group required recovery on 1 or more occasions. Control animals were fasted and given saline injections. No animals suffered from seizures.

Behavioral Procedures

Cognition was assessed by novel object recognition (NOR) and spontaneous alternation tests (28; 29), at least 3 days after the last hypoglycemic episode (Figure S1A). Beckman Activity Box and Open field maze tasks were also made to exclude any confounding effects of altered activity or anxiety.

Novel object recognition

A simple hippocampal-mediated task based on the innate tendency of rodents to seek novelty was used (28). The primary outcome measure was the discrimination index (D3), calculated as the total time spent exploring the familiar objects/ total time spent exploring the novel object, for short-term (10 min) and long-term (24hr) memory.

Spontaneous alternation

This was used to test spatial working memory as described previously (29). Memory was assessed as a percent 4/5 alternation with an alternation counted when all four arms are visited within a span of five arm entries (29).

Activity box

Locomotor activity was assessed using a Beckman Activity Box. Animals, habituated to the box for 4 days, were placed into the box and allowed to explore freely for 15 minutes. Mobile, active and static counts were recorded.

Open field maze

Activity was recorded on film and later analysed by two independent scorers for total time spent within the inner and outer zones of the maze. Activity is presented as % total time spent in each zone.

Biochemical analyses

On completion of behavioral testing, animals were killed humanely, brain tissue dissected and flash frozen in liquid nitrogen for subsequent biochemical analyses.

Lipid peroxidation

The concentration of malondialdehyde (MDA) was determined in hippocampus using the thiobarbituric acid reactive substances (TBARS) assay as described in (30), and adapted for a 96-well plate format. The amount of MDA in the samples was determined spectrophotometrically at 532 nm and concentration determined from a standard curve. All samples were assayed in duplicate.

Protein carbonylation

Levels of carbonylated protein within the hippocampus were measured by ELISA (Caymen Chemicals Ltd). Protein carbonyl concentration was calculated using the following equation: Protein carbonyl (nmol/ml)=[(CA)/(*0.011 mM-1)](500 ml/200 ml), where CA is equal to the corrected absorbance (average absorbance of controls – average absorbance of samples).

Pro-inflammatory protein array

The total protein levels of a panel of inflammatory cytokines (IFNγ, IL-10, IL-12p70, IL-1β□IL-2, IL-4, IL-5, IL-6, KC/GRO and TNFα□were measured using the V-Plex Pro-inflammatory Panel (mouse) kit (MSD). Hippocampal homogenates (50µg) were assayed in duplicate and values presented relative to their appropriate control group (Con or *Nrf2*$^{-/-}$ respectively).

RNA extraction and PCR

Total RNA was extracted from hippocampal tissue using TRIzol® reagent (Invitrogen). Reverse transcription was performed with 1 ng RNA using SuperScript® III First Strand Synthesis system for RT (Invitrogen). Real-time PCR was performed using Taqman gene expression assays for the following genes: Nrf2 and Nrf2 target-genes (*Hmox1, Nqo1, Gsta1, Txn1, Txrnd1* and *Srxn1*(31)), the inflammatory genes Il-1β, Il-6, Tnfα, and Nos2, and house-keeping genes (Applied Biosystems; See Supplementary Table 1 for full details of primer/probe sequences). All samples were performed in triplicate and normalized to Actin. Values are expressed as a fold-change relative to Control.

Statistical analysis

Data were analyzed using SPSS version 18. Multivariate analysis of variance (ANOVA) was used to compare groups with treatment (TID) or genotype (*Nrf2*$^{-/-}$) and Hypo as between subject variables. Post-hoc analysis was performed using Tukey's multiple comparisons test. Data are expressed as mean values ±SEM. Statistical significance was set at $p<0.05$.

RESULTS

Recurrent hypoglycemia in wild-type and *Nrf2*$^{-/-}$ mice with or without T1D

As expected, blood glucose levels were higher in wild-type T1D compared to control animals over the 12-week study (Figure 1A; T1D x week $F(3.74,93.48)=35.89$; $p<0.05$). However, the level of hypoglycemia achieved was comparable between T1D and Control groups, (Figure 1B; Average glucose, Control=2.9±0.3 vs. TID=3.0±0.4 mmol/l; $t=0.088$; $p=0.97$) and did not change over the duration of the experiment (T1D $F(1,26)=0.621$; $p=0.45$).

Similarly, T1D *Nrf2*$^{-/-}$ mice had significantly higher fasting glucose levels when compared to non-diabetic *Nrf2*$^{-/-}$ animals for the duration of the study (Figure 1C ; T1D x week $F(3.66,36.66)= 21.53$; <0.01) but the degree of hypoglycemia achieved between T1D *Nrf2*$^{-/-}$ and *Nrf2*$^{-/-}$ animals was comparable (Figure 1D. Average glucose, *Nrf2*$^{-/-}$ =2.4±0.3 vs. TID *Nrf2*$^{-/-}$=3.1±0.6 mmol/l; $t=0.062$, $p=0.95$).

Recurrent hypoglycemia impairs cognitive function in T1D wild-type and *Nrf2*$^{-/-}$ mice

The most significant cognitive defect found was in long-term memory as assessed by the NOR task in T1D + Hypo mice (Figure 2A; T1D x Hypo; $F(1,30)=5.936$, $p<0.05$). A less marked cognitive defect at this time point was also found in T1D mice (Figure 2A; T1D $F(1,30)=52.40$, $p<0.05$), but not in control or control + Hypo animals. These differences were not due to a reduction in time spent exploring the objects (effect of T1D and Hypo both p=ns). Performance on the NOR task after a 10min interval was intact in all groups (main effects T1D and Hypo on D3 indices and Exploration time; all p=ns), indicating that both the ability to learn the task and short-term memory for objects were intact

On the spontaneous alternation task, T1D + Hypo animals were also most affected, demonstrating a reduction in percentage alternations ([Total alternations/ total entries-4)*100] Figure 2B; T1D x Hypo $(1,15)= 5.71$; $p<0.01$) and fewer total alternations (T1D x Hypo$F(1,15)=7.734$; $p<0.01$; Average total alternations; Control 46.29±3.66, Control + Hypo 48.36 ±3.63, T1D 47.10±4.67, T1D + Hypo 41.67±3.80). In contrast, control animals receiving Hypo showed a trend towards enhanced % alternation (Figure 2B; $F(1,15)=3.04$; $p=0.10$). There was no effect of T1D alone on the total number of entries ($F(1,30)=2.92$; p=ns).

As with wild-types, all *Nrf2*$^{-/-}$animals performed the short term NOR task above chance levels (D3 index of ≥0.2; F<1, p=ns), and as before there were no differences between groups in discrimination indices and time spent exploring the objects ((D3 indices and Exploration time; all F<1, p=ns). However, after 24 hours, only control *Nrf2*$^{-/-}$ animals were able to perform the task with both Hypo, T1D and Hypo+T1D markedly impairing long-term memory (Figure 2C; Hypo $F(1,25)=12.09$, $p<0.01$; T1D $F(1,25)=6.95$, $p<0.05$); T1D x Hypo; $F(1,25)=6.24$, $p<0.05$). These differences were not due to a reduction in time spent exploring the objects (Exploration time; all F<1, p=ns).

In both non-diabetic and T1D *Nrf2*$^{-/-}$ animals Hypo resulted in impaired working memory ([Total alternations/ total entries-4)*100] Figure 2D; Hypo $F(1,25)=18.18$; $p<0.01$; F<1 for the effect of T1D and T1D x Hypo). This impairment was accompanied by a significant reduction in the total number of alternations (Hypo $F(1,25)=9.22$, $p<0.01$; T1D $F(1,25)=17.28$, $p<0.01$; F<1, p=ns for effect of T1D x Hypo; Average total alternations; *Nrf2*$^{-}$

$^{-/-}$ 28.35±2.78, *Nrf2*$^{-/-}$+ Hypo 21.00±2.80, T1D *Nrf2*$^{-/-}$24.86±4.11, T1D *Nrf2*$^{-/-}$ + Hypo 10.14±1.83). As with wild-type mice, there was no difference in total number of entries (T1D F(1,25)=4.12 p=0.776; F<1, p=ns for effect of Hypo and T1D x Hypo).

Assessments of locomotor activity and anxiety on the open field maze (data not shown), did not differ between any groups [active, mobile, or static counts (all F<1); time spent in central inner zone or the outer peripheral zone (all F<1)]

Recurrent hypoglycemia and chronic hyperglycemia act synergistically to activate an oxidative stress response

To explore the mechanisms underpinning the cognitive impairments demonstrated in working and long-term memory, transcript abundance of transcription factor Nrf2 as well as that of its target genes *Hmox1, Nqo1, Gsta1, Txn1, Txnrd1* and *Srxn1*were measured in the hippocampus of each animal model. Significant changes in gene expression were seen in response to T1D and Hypo in both wild type and *Nrf2*$^{-/-}$ animals (Table 1). Both T1D and T1D+Hypo increased mRNA for *Hmox1* and *Nqo1* in all animals, while a marked increase in Nrf2 expression was seen in the hippocampus of T1D+Hypo wild type mice. As expected, the expression of other Nrf2-regulated genes (*Gsta1, Txn1, Txnrd1* and *Srxn1*) appeared to be significantly diminished in all *Nrf2*$^{-/-}$mice irrespective of the metabolic model applied. Interestingly, mRNA for *Gsta1* was significantly decreased in response to Hypo in T1D and non-diabetic WT mice, and this effect was lost in *Nrf2*$^{-/-}$mice (Table 1).

Recurrent hypoglycemia and chronic hyperglycemia act synergistically to provoke ROS-induced cell damage in the hippocampus

To examine for evidence of ROS induced cellular damage, levels of lipid peroxidation and protein carbonylation were determined in hippocampal homogenates. Hypo in both non-diabetic and T1D mice increased lipid peroxidation within the hippocampus (Figure 3A; Hypo F(1,30)=8.36, p<0.05). Moreover, there was a T1D x Hypo interaction, indicating an additional stimulus to lipid peroxidation in T1D mice exposed to repeated hypoglycemia (T1D x Hypo F(1,28)=6.24 p<0.05). Protein carbonylation, an irreversible process resulting from exposure to ROS and an indicator of severe oxidative damage (32), was significantly elevated in the hippocampus only in T1D mice exposed to Hypo (Figure 3B; T1D x Hypo F(1,28)=4.35; p<0.05).

In *Nrf2*$^{-/-}$mice, hippocampal lipid peroxidation (Figure 3C) was increased by Hypo (F(1,25)=76.86; p<0.01), and greatest in T1D *Nrf2*$^{-/-}$ animals following Hypo (T1D x Hypo F(1,25)=5.07; p<0.05). Protein carbonylation was increased with Hypo (Figure 3D; Hypo F(1,25)=38.08; p<0.01), but not T1D (F<1, p=ns for T1D and T1D x Hypo).

Recurrent hypoglycemia and chronic hyperglycemia act synergistically to induce an inflammatory response within the hippocampus

To determine whether Hypo in T1D elicited an inflammatory response, cytokine protein levels were measured within hippocampal homogenates. In wild-type mice, IL-1β, IL-2, IL-4, IL-6 and TNFα were significantly enhanced in T1D animals whereas exposure to Hypo

significantly increased levels of IL-12p70 and IL-5 (Table 2; all, $p<0.05$). The combination of Hypo + T1D resulted in a marked stimulus to the production of IL-1β, IL-2, TNFα, IL-4, IL-6, IL12p70, IL-5 (Table 2; all $p<.01$)

A similar, but more marked pattern in cytokine release was seen in *Nrf2*$^{-/-}$ animals. IL-1β, IL-2, TNFα, IL-4, IL-6, IL12p70, and IL-5 levels were increased in T1D, whereas IL-6, IL-12p70 and IL-5 were significantly increased following Hypo (Table 2; all $p< 0.05$). Again, in *Nrf2*$^{-/-}$mice the combination of Hypo + T1D resulted in a marked stimulus to the production of IL-1β, IL-2, TNFα, IL-4, IL-6, IL12p70, IL-5 (Table 2; all $p<0.001$). Interestingly, levels of all cytokines measured were increased even in untreated *Nrf2*$^{-/-}$mice, and amplified following each metabolic stimulus, although the pattern of change was nearly identical to that seen in wild-type mice.

The transcript abundance of the inflammatory genes *IL-1β*, *IL-6*, *Tnfα*, and *Nos2* were also checked and showed a similar pattern of change to protein levels of each cytokine, although these increases only reached significance in T1D and T1D+Hypo *Nrf2*$^{-/-}$ animals (Supplementary Table 2).

DISCUSSION

In this manuscript we show for the first time in a healthy insulin-treated animal model of T1D that recurrent non-severe hypoglycemia may have a significant impact on neuronal integrity and function. We show that 8 episodes of non-severe hypoglycemia in T1D over a 4-week period induces significant defects in memory consolidation and working memory, and that this is associated with persisting biochemical evidence of oxidative stress and inflammation in the hippocampus. Interestingly, protein synthesis, which is considered to play an integral role in memory consolidation, is disrupted at many levels by oxidative stress (33). In contrast and consistent with the work of others, recurrent hypoglycemia in non-diabetic rodents did not impair cognitive function and may even have improved cognitive performance (34). The key role for oxidative stress in inducing cognitive impairment was further illustrated in the demonstration of a marked amplification of the hippocampal oxidative stress and inflammatory response in mice lacking the Nrf2 transcription factor (following recurrent hypoglycemia).

Pre-clinical and clinical studies suggest that marked glycemic variability maybe detrimental to humans (22; 35-37). In the current study, glucose variability is represented by three principal metabolic states, namely chronic hyperglycemia, acute hypoglycemia, and 'recovery' from acute hypoglycemia. Hypoglycemia can cause oxidative stress (20) and inflammation (21), and hypoglycemia-mediated ROS production can induce apoptosis (38; 39). However, the fact that no impairment in cognitive performance was seen following Hypo in wild-type mice implies that the intrinsic antioxidant capacity, coupled with the oxidative stress response orchestrated by Nrf2, is ordinarily sufficient to protect the neuron from the consequences of moderate glucose deprivation. Consistent with this, recurrent hypoglycemia despite recovery to normal glucose levels, resulted in cognitive impairment in *Nrf2*$^{-/-}$ mice. More recent studies in hippocampal slice preparations have shown that oxidative stress and

neuronal death occur primarily in the recovery period from hypoglycemia during glucose reperfusion, and that the extent of oxidative stress correlates with the rise in glucose during recovery (13). In our model, glucose levels post-hypoglycemia were >16mmol/l and therefore glucose reperfusion into neurons that have experienced prolonged energy deprivation (as in ischemia reperfusion injury) may be the major contributor to oxidative stress. But Nrf2 protein levels and function are decreased in humans and rodents with diabetes (40; 41), therefore the Nrf2-mediated defense mechanism may also be insufficient to prevent oxidative damage resulting from non-severe hypoglycemia in T1D. Neuronal damage secondary to severe hypoglycemia is exacerbated in T1D rats compared with non-diabetic controls (14). Taken together, our data provide robust evidence that recurrent non-severe hypoglycemia in T1D can provoke sufficient oxidative stress to induce a local inflammatory response and result in neuronal dysfunction, but cannot differentiate between potential effects of hypoglycemia *per se* or recovery to hyperglycemic levels. Future studies will be required to address this clinically important question.

Chronic hyperglycemia increases the production of ROS through mechanisms such as glucose auto-oxidation and non-enzymatic protein glycation (23), and lowers antioxidant defense mechanisms (24; 25) as well as serum free-radical trapping capacity (23). In the present study, both T1D wild-type and T1D *Nrf2*$^{-/-}$mice demonstrated impairments in memory consolidation. Neither model had tissue evidence of increased lipid peroxidation or protein carbonylation suggesting no significant oxidative damage, but both models had significantly increased levels of *Nrf2, Hmox-1, Nqo-1* and the inflammatory cytokines *IL-1β, IL-2, IL-4, IL-6* and *Tnfα*. Intriguingly, loss of Nrf2 amplified both the inflammatory response and cognitive defect. It is recognized that inflammatory responses are exacerbated in *Nrf2*$^{-/-}$ mice (42; 43), and such an outcome is consistent in humans and rodents with diabetes where Nrf2 levels and activity are diminished (40; 41). This is consistent with a recognized hierarchical response to oxidative stressors whereby modest levels of ROS activate an Nrf2-orchestrated adaptation whereas higher levels of ROS stimulate NF-κB and AP-1 to provide an additional defense mechanism (44). In this case, *Nrf2*$^{-/-}$mice would be anticipated to have higher levels of ROS under both hypo- and also hyperglycemia conditions leading to a pro-inflammatory state. Recent studies indicating a role for Nrf2 agonists in the treatment of diabetic nephropathy (43) and cardiomyopathy (45) that are associated with chronic hyperglycemia would be consistent with this possibility. The results of the present study would suggest that chronic hyperglycemia induces a pro-inflammatory condition through Nrf2-dependent and independent mechanisms.

A critical and novel finding in this paper is the important role of the transcription factor Nrf2 in initiating the oxidative stress response to hypoglycemia. Nrf2 is a transcription factor that dictates the intrinsic antioxidant capacity of cells under normal physiological conditions and also directs adaptation to oxidative stress. The activity of Nrf2 is itself regulated through a complex transcriptional/epigenetic and post-translational network in a manner that ensures its function increases during redox perturbation, inflammation and nutrient/energy fluxes, thereby enabling the factor to orchestrate adaptive responses to diverse forms of stress [for a review, see (26)]. Previously, Johnson and colleagues have reported that *Nrf2*$^{-/-}$ mice are

significantly more sensitive to kainate neuronal toxicity than their wild-type counterparts, and used microarray analysis to show this was associated with markedly reduced expression in the hippocampus of *aldehyde oxidase 1, Gstm1, Gstm3, peroxiredoxin 1* (*Prdx1*) and *Prdx2* (46). In our study, the responses of $Nrf2^{+/+}$ and $Nrf2^{-/-}$ mice to T1D and Hypo were examined in separate experiments and we therefore cannot strictly compare gene expression profiles between wild-type and knockout mice. Nevertheless, it was apparent from our gene expression analyses that large differences exist between the expression of *Gsta1, Gstm1* and *Srxn1*in the hippocampus of in $Nrf2^{+/+}$ and $Nrf2^{-/-}$ mice. In the future, it will be desirable to examine simultaneously the influence that loss of Nrf2 and genetic upregulation of Nrf2 (caused by diminished expression of Kelch-like ECH-associated protein 1 (Keap1), a negative regulator of Nrf2) has on gene expression and cognitive function following T1D and Hypo. It is notable that we found the levels of mRNA for Nrf2 were significantly upregulated by T1D, and very dramatically (by 12-fold) by T1D + Hypo. These results are consistent with a recent report that the protein levels of Nrf2 are increased in the diabetic wounds of humans and mice, and that pharmacological activation of Nrf2 promotes wound healing in T1D mice (47). Together with the heightened inflammation in T1D and T1D + Hypo$Nrf2^{-/-}$ mice relative to their wild-type counterparts, these findings suggest that one of the functions of Nrf2 is to act as a "brake" to control inflammation. Our current results illustrate the critical importance of this function of Nrf2: indeed, inflammation is controlled and cognitive function is largely preserved in wild-type animals subjected to hypoglycemia, whereas inflammation is enhanced and cognitive function is severely impaired in their $Nrf2^{-/-}$ counterparts.

In conclusion, our study supports the hypothesis that recurrent moderate hypoglycemia in T1D may have long-term consequences on cognitive function. Our findings suggest that chronic hyperglycemia, recurrent hypoglycemia, and glucose reperfusion following hypoglycemia in T1D interact synergistically to induce pathological oxidative stress in vulnerable brain regions such as the hippocampus. Whether hypoglycemia *per se* or glucose recovery from hypoglycemia is the major contributor to oxidative damage in T1D cannot be determined from our studies although pre-clinical research suggests the recovery period is key. This has implications for clinical practice where treatment of hypoglycemia often leads to marked rebound hyperglycemia. Moreover, we provide evidence that the transcription factor Nrf2 may be integral to neuronal protection against oxidative stress during and following hypoglycemia, and in response to chronic hyperglycemia. This raises the possibility of targeting Nrf2 in developing therapies designed to prevent cellular damage in diabetes induced by recurrent hypoglycemia.

Acknowledgements

Funding: This work was supported by an award from the University of Dundee/ Wellcome Trust Translational Medical Research Fund (RJM), Diabetes UK (12/0004531) and Juvenile Diabetes Research Foundation (5-2011-464).

Author Contributions: A.D.M. designed and performed experiments and wrote manuscript. J.R.G. performed experiments. A.T.D-K. contributed to discussion and reviewed /edited manuscript. J.D.H. contributed to discussion and reviewed /edited manuscript. M.L.J.A. contributed to discussion and reviewed /edited manuscript. J.S. contributed to design of experiments and discussion and research reviewed /edited manuscript. R.J.M designed experiments and wrote manuscript. R.J.M. is overall guarantor for the contents of the article.

References

1. McCrimmon RJ, Sherwin RS: Hypoglycemia in type 1 diabetes. Diabetes 2010;59:2333-2339

2. McCrimmon RJ, Frier BM: Hypoglycaemia: the most feared complication of insulin therapy. Diabete & Metabolisme 1994;20:503-512

3. Chalmers J, Risk MT, Kean DM, Grant R, Ashworth B, Campbell IW: Severe amnesia after hypoglycemia. Clinical, psychometric, and magnetic resonance imaging correlations. Diabetes Care 1991;14:922-925

4. Fujioka M, Okuchi K, Hiramatsu KI, Sakaki T, Sakaguchi S, Ishii Y: Specific changes in human brain after hypoglycemic injury. Stroke; a journal of cerebral circulation 1997;28:584-587

5. McCrimmon RJ, Ryan CM, Frier BM: Diabetes and cognitive dysfunction. Lancet 2012;379:2291-2299

6. Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications Study Research G, Jacobson AM, Musen G, Ryan CM, Silvers N, Cleary P, Waberski B, Burwood A, Weinger K, Bayless M, Dahms W, Harth J: Long-term effect of diabetes and its treatment on cognitive function. New England Journal of Medicine 2007;356:1842-1852

7. Hershey T, Perantie DC, Warren SL, Zimmerman EC, Sadler M, White NH: Frequency and timing of severe hypoglycemia affects spatial memory in children with type 1 diabetes. Diabetes care 2005;28:2372-2377

8. Ryan CM, Atchison J, Puczynski S, Puczynski M, Arslanian S, Becker D: Mild hypoglycemia associated with deterioration of mental efficiency in children with insulin-dependent diabetes mellitus. The Journal of pediatrics 1990;117:32-38

9. Patino-Fernandez AM, Delamater AM, Applegate EB, Brady E, Eidson M, Nemery R, Gonzalez-Mendoza L, Richton S: Neurocognitive functioning in preschool-age children with type 1 diabetes mellitus. Pediatric diabetes 2010;11:424-430

10. Perantie DC, Wu J, Koller JM, Lim A, Warren SL, Black KJ, Sadler M, White NH, Hershey T: Regional brain volume differences associated with hyperglycemia and severe hypoglycemia in youth with type 1 diabetes. Diabetes care 2007;30:2331-2337

11. Perantie DC, Koller JM, Weaver PM, Lugar HM, Black KJ, White NH, Hershey T: Prospectively determined impact of type 1 diabetes on brain volume during development. Diabetes 2011;60:3006-3014

12. Auer RN, Wieloch T, Olsson Y, Siesjo BK: The distribution of hypoglycemic brain damage. Acta neuropathologica 1984;64:177-191

13. Suh SW, Gum ET, Hamby AM, Chan PH, Swanson RA: Hypoglycemic neuronal death is triggered by glucose reperfusion and activation of neuronal NADPH oxidase. J Clin Invest 2007;117:910-918

14. Bree AJ, Puente EC, Daphna-Iken D, Fisher SJ: Diabetes increases brain damage caused by severe hypoglycemia. American journal of physiology Endocrinology and metabolism 2009;297:E194-201

15. Won SJ, Yoo BH, Kauppinen TM, Choi BY, Kim JH, Jang BG, Lee MW, Sohn M, Liu J, Swanson RA, Suh SW: Recurrent/moderate hypoglycemia induces hippocampal dendritic injury, microglial activation, and cognitive impairment in diabetic rats. Journal of neuroinflammation 2012;9:182

16. Yamada KA, Rensing N, Izumi Y, De Erausquin GA, Gazit V, Dorsey DA, Herrera DG: Repetitive hypoglycemia in young rats impairs hippocampal long-term potentiation. Pediatr Res 2004;55:372-379

17. McNay EC, Williamson A, McCrimmon RJ, Sherwin RS: Cognitive and neural hippocampal effects of long-term moderate recurrent hypoglycemia. Diabetes 2006;55:1088-1095

18. Puente EC, Silverstein J, Bree AJ, Musikantow DR, Wozniak DF, Maloney S, Daphna-Iken D, Fisher SJ: Recurrent moderate hypoglycemia ameliorates brain damage and cognitive dysfunction induced by severe hypoglycemia. Diabetes 59:1055-1062

19. Butterfield DA, Di Domenico F, Barone E: Elevated risk of type 2 diabetes for development of Alzheimer disease: a key role for oxidative stress in brain. Biochim Biophys Acta 2014;1842:1693-1706

20. Singh P, Jain A, Kaur G: Impact of hypoglycemia and diabetes on CNS: correlation of mitochondrial oxidative stress with DNA damage. Molecular and cellular biochemistry 2004;260:153-159

21. Wright RJ, Newby DE, Stirling D, Ludlam CA, Macdonald IA, Frier BM: Effects of acute insulin-induced hypoglycemia on indices of inflammation: putative mechanism for aggravating vascular disease in diabetes. Diabetes care 2010;33:1591-1597

22. Ceriello A, Novials A, Ortega E, La Sala L, Pujadas G, Testa R, Bonfigli AR, Esposito K, Giugliano D: Evidence that hyperglycemia after recovery from hypoglycemia worsens endothelial function and increases oxidative stress and inflammation in healthy control subjects and subjects with type 1 diabetes. Diabetes 2012;61:2993-2997

23. Dominguez C, Ruiz E, Gussinye M, Carrascosa A: Oxidative stress at onset and in early stages of type 1 diabetes in children and adolescents. Diabetes care 1998;21:1736-1742

24. Marra G, Cotroneo P, Pitocco D, Manto A, Di Leo MA, Ruotolo V, Caputo S, Giardina B, Ghirlanda G, Santini SA: Early increase of oxidative stress and reduced antioxidant defenses in patients with uncomplicated type 1 diabetes: a case for gender difference. Diabetes care 2002;25:370-375

25. Vucic M, Gavella M, Bozikov V, Ashcroft SJ, Rocic B: Superoxide dismutase activity in lymphocytes and polymorphonuclear cells of diabetic patients. European journal of clinical chemistry and clinical biochemistry : journal of the Forum of European Clinical Chemistry Societies 1997;35:517-521

26. Hayes JD, Dinkova-Kostova AT: The Nrf2 regulatory network provides an interface between redox and intermediary metabolism. Trends Biochem Sci 2014;39:199-218

27. Chowdhry S, Nazmy MH, Meakin PJ, Dinkova-Kostova AT, Walsh SV, Tsujita T, Dillon JF, Ashford ML, Hayes JD: Loss of Nrf2 markedly exacerbates nonalcoholic steatohepatitis. Free radical biology & medicine 2010;48:357-371

28. Langston RF, Wood ER: Associative recognition and the hippocampus: differential effects of hippocampal lesions on object-place, object-context and object-place-context memory. Hippocampus 2010;20:1139-1153

29. McNay EC, Fries TM, Gold PE: Decreases in rat extracellular hippocampal glucose concentration associated with cognitive demand during a spatial task. Proceedings of the National Academy of Sciences of the United States of America 2000;97:2881-2885

30. Mihara M, Uchiyama M: Determination of malonaldehyde precursor in tissues by thiobarbituric acid test. Analytical biochemistry 1978;86:271-278

31. Gorrini C, Harris IS, Mak TW: Modulation of oxidative stress as an anticancer strategy. Nature reviews Drug discovery 2013;12:931-947

32. Jacob KD, Noren Hooten N, Trzeciak AR, Evans MK: Markers of oxidant stress that are clinically relevant in aging and age-related disease. Mechanisms of ageing and development 2013;134:139-157

33. Shenton D, Smirnova JB, Selley JN, Carroll K, Hubbard SJ, Pavitt GD, Ashe MP, Grant CM: Global translational responses to oxidative stress impact upon multiple levels of protein synthesis. J Biol Chem 2006;281:29011-29021

34. McNay EC, Teske JA, Kotz CM, Dunn-Meynell A, Levin BE, McCrimmon RJ, Sherwin RS: Long-term, intermittent, insulin-induced hypoglycemia produces marked obesity without hyperphagia or insulin resistance: a model for weight gain with intensive insulin therapy. Am J Physiol Endocrinol Metab 2013;304:E131-138

35. Rehni AK, Nautiyal N, Perez-Pinzon MA, Dave KR: Hyperglycemia / hypoglycemia-induced mitochondrial dysfunction and cerebral ischemic damage in diabetics. Metabolic brain disease 2014;

36. Brownlee M: The pathobiology of diabetic complications: a unifying mechanism. Diabetes 2005;54:1615-1625

37. Russo VC, Higgins S, Werther GA, Cameron FJ: Effects of fluctuating glucose levels on neuronal cells in vitro. Neurochemical research 2012;37:1768-1782

38. Manna SK, Zhang HJ, Yan T, Oberley LW, Aggarwal BB: Overexpression of manganese superoxide dismutase suppresses tumor necrosis factor-induced apoptosis and activation of nuclear transcription factor-kappaB and activated protein-1. The Journal of biological chemistry 1998;273:13245-13254

39. Nomura K, Imai H, Koumura T, Arai M, Nakagawa Y: Mitochondrial phospholipid hydroperoxide glutathione peroxidase suppresses apoptosis mediated by a mitochondrial death pathway. The Journal of biological chemistry 1999;274:29294-29302

40. Tan Y, Ichikawa T, Li J, Si Q, Yang H, Chen X, Goldblatt CS, Meyer CJ, Li X, Cai L, Cui T: Diabetic downregulation of Nrf2 activity via ERK contributes to oxidative stress-induced insulin resistance in cardiac cells in vitro and in vivo. Diabetes 2011;60:625-633

41. He HJ, Wang GY, Gao Y, Ling WH, Yu ZW, Jin TR: Curcumin attenuates Nrf2 signaling defect, oxidative stress in muscle and glucose intolerance in high fat diet-fed mice. World journal of diabetes 2012;3:94-104

42. Tebay LE, Robertson H, Durant ST, Vitale SR, Penning TM, Dinkova-Kostova AT, Hayes JD: Mechanisms of activation of the transcription factor Nrf2 by redox stressors, nutrient cues, and energy status and the pathways through which it attenuates degenerative disease. Free Radic Biol Med 2015;88:108-146

43. Jiang T, Huang Z, Lin Y, Zhang Z, Fang D, Zhang DD: The protective role of Nrf2 in streptozotocin-induced diabetic nephropathy. Diabetes 2010;59:850-860

44. Hamanaka RB, Chandel NS: Mitochondrial reactive oxygen species regulate cellular signaling and dictate biological outcomes. Trends Biochem Sci 2010;35:505-513

45. Wang Y, Sun W, Du B, Miao X, Bai Y, Xin Y, Tan Y, Cui W, Liu B, Cui T, Epstein PN, Fu Y, Cai L: Therapeutic effect of MG-132 on diabetic cardiomyopathy is associated with its suppression of proteasomal activities: roles of Nrf2 and NF-kappaB. Am J Physiol Heart Circ Physiol 2013;304:H567-578

46. Kraft AD, Lee JM, Johnson DA, Kan YW, Johnson JA: Neuronal sensitivity to kainic acid is dependent on the Nrf2-mediated actions of the antioxidant response element. Journal of neurochemistry 2006;98:1852-1865

47. Long M, Rojo de la Vega M, Wen Q, Bharara M, Jiang T, Zhang R, Zhou S, Wong PK, Wondrak GT, Zheng H, Zhang DD: An Essential Role of NRF2 in Diabetic Wound Healing. Diabetes 2016;65:780-793

Figure 1. Physiological profile of diabetic and non-diabetic WT and *Nrf2*$^{-/-}$ mice exposed to recurrent hypoglycemia. A and C: Body weight was comparable between experimental groups and unaltered by either genotype or recurrent hypoglycemia. B and D: Example of typical insulin induced hypoglycemic event in WT and *Nrf2*$^{-/-}$ animals with a comparable level of hypoglycemia achieved despite *Nrf2*$^{-/-}$ animals receiving significantly lower insulin doses (starting dose 0.75mU/g WT control vs. 0.5mU/g *Nrf2*$^{-/-}$ animals). Control (Con), Con + Hypo, T1D and T1D + Hypo all n=8 per group. *Nrf2*$^{-/-}$, *Nrf2*$^{-/-}$+ Hypo, T1D *Nrf2*$^{-/-}$ and T1D *Nrf2*$^{-/-}$ + Hypo all n=7-8 per group. Results represent mean values ± SEM. Data were analyzed by 2-way ANOVA with T1D and Hypo as between subject factors followed by Tukey's post hoc test *p<0.05, **p<0.01 Control or *Nrf2*$^{-/-}$vs T1D or T1D *Nrf2*$^{-/-}$, ♯p<0.05, ♯♯p<0.01 Hypo vs. No Hypo.

Figure 2. Recurrent hypoglycemia (Hypo) in T1D mice impairs cognitive performance in novel-object recognition (NOR) and Spontaneous Alternation tasks. A: Discrimination index (D3) demonstrating that T1D mice are significantly impaired when tested in the 24hr NOR task. This is exacerbated in T1D animals following Hypo. B: Mean 4/5 alternation performance on a closed arm plus maze, expressed as a percentage of possible alternations is significantly reduced in T1D animals following Hypo. C: *Nrf2*$^{-/-}$ and T1D *Nrf2*$^{-/-}$ mice following Hypo were significantly impaired when tested in the 24hr NOR task, and D: Mean 4/5 alternation performance on a closed arm plus maze, expressed as a percentage of possible alternations is significantly reduced in *Nrf2*$^{-/-}$+ Hypo and T1D *Nrf2*$^{-/-}$+ Hypo animals when compared to their control (no hypo) counterparts. Control (Con), Con + Hypo, T1D and T1D + Hypo all n=8 per group. *Nrf2*$^{-/-}$, *Nrf2*$^{-/-}$+ Hypo, T1D *Nrf2*$^{-/-}$ and T1D *Nrf2*$^{-/-}$ + Hypo all n=7-8 per group. Results represent mean values ± SEM. Data were analyzed by 2-way ANOVA with T1D and Hypo as between subject factors followed by Tukey's post hoc test *p<0.05 Control or *Nrf2*$^{-/-}$vs T1D or T1D *Nrf2*$^{-/-}$, ♯p<0.05, ♯♯p<0.01 Hypo vs. No Hypo

Figure 3. Recurrent hypoglycemia in T1D is associated with increased markers of oxidative damage. The levels of lipid peroxidation were determined using the thiobarbituric acid reactive substances (TBARS) assay and protein carbonylation by ELISA. A: The levels of lipid peroxidation and B: protein carbonylation were significantly elevated following Hypo and further enhanced in T1D mice. The levels of C. Lipid peroxidation and D: protein carbonylation were augmented following Hypo in *Nrf2*$^{-/-}$ mice and enhanced further in T1D *Nrf2*$^{-/-}$ + Hypo animals. Control (Con), Con + Hypo, T1D and T1D + Hypo all n=8 per group. *Nrf2*$^{-/-}$, *Nrf2*$^{-/-}$+ Hypo, T1D *Nrf2*$^{-/-}$ and T1D *Nrf2*$^{-/-}$ + Hypo all n=7-8 per group. Results represent mean values ± SEM. Data were analyzed by 2-way ANOVA with T1D and Hypo as between subject factors followed by Tukey's post hoc test *p<0.05 Control or *Nrf2*$^{-/-}$vs T1D or T1D *Nrf2*$^{-/-}$, ♯p<0.05, ♯♯p<0.01 Hypo vs. No Hypo.

Tables 1. Recurrent hypoglycemia and T1D in both Wild-type and Nrf2$^{-/-}$ mice is associated with modulation of genes involved in mediating anti-oxidant and redox systems. mRNA was extracted and processed for real-time PCR to evaluate changes in gene expression of Hemeoxygenase 1 (Hmox1), Nrf2, NAD(P)H:quinoneoxidoreductase (Nqo1), Thioredoxinreductase 1 (Txnrd1), Sulfiredoxin 1 (Srxn1), Thioredoxin 1 (Txn1) and Glutathione*S*-transferase Alpha subunit 1 (Gsta1). Control (Con), Con + Hypo, T1D and T1D + Hypo all n=8 per group. *Nrf2*$^{-/-}$, *Nrf2*$^{-/-}$+ Hypo, T1D *Nrf2*$^{-/-}$ and T1D *Nrf2*$^{-/-}$ + Hypo all n=7-8 per group. Results represent mean values ± SEM. Data were analyzed within genotype by 2-way ANOVA with T1D and Hypo as between subject factors followed by Tukey's post hoc test *p<0.05, **p<0.01 Control or *Nrf2*$^{-/-}$vs T1D or T1D *Nrf2*$^{-/-}$, ♯p<0.05, ♯♯p<0.01 Hypo vs. No Hypo

Gene	Control	Control + Hypo	T1D	T1D + Hypo		Nrf2$^{-/-}$	Nrf2$^{-/-}$ + Hypo	T1D Nrf2$^{-/-}$	T1D Nrf2$^{-/-}$ + Hypo
Hmox-1	1.00±0.28	1.15±0.14	1.34±0.14*	1.88±0.55*♯		0.71±0.07	0.49±0.08	1.05±0.11**	1.15±0.11**♯
Nrf2	1.00±0.15	1.14±0.18	1.86±0.18*	12.13±0.10*♯		n/d	n/d	n/d	n/d
Nqo1	1.00±0.35	1.53±0.17	2.11±0.28*	3.20±0.44*♯		1.94±0.13	1.78±0.12	2.18±0.31*	2.74±0.24*♯
Txnrd1	1.00±0.19	1.07±0.10	1.08±0.10	1.09±0.13		0.79±0.05	0.95±0.12	0.60±0.08	0.59±0.05
Srxn1	1.00±0.09	0.94±0.06	1.06±0.03	1.13±0.14		0.58±0.10	0.71±0.16	0.43±0.05	0.48±0.04
Txn1	1.00±0.16	0.96±0.11	1.28±0.19	1.32±0.23		0.82±0.06	0.74±0.11	0.73±0.11	0.76±0.11
Gsta1	1.00±0.13	0.50±0.76♯	1.00±0.06	0.53±0.16♯		0.11±0.04	0.22±0.07	0.10±0.04	0.10±0.04

Table 2. Recurrent hypoglycemia and T1D induce inflammation within the hippocampus in both WT T1D animals and *Nrf2*$^{-/-}$animals respectively. Levels of a number of common inflammatory cytokines were measured within hippocampal homogenates by ELISA. Con, Con + Hypo, T1D and T1D + Hypo all n=8 per group. *Nrf2*$^{-/-}$, *Nrf2*$^{-/-}$+ Hypo, T1D *Nrf2*$^{-/-}$ and T1D *Nrf2*$^{-/-}$+ Hypo all n=7-8 per group. Results represent mean values ± SEM. Data were analyzed within genotype by 2-way ANOVA with T1D and Hypo as between subject factors followed by Tukey's post hoc test * p<0.05, test *p<0.05, **p<0.01 Control or *Nrf2*$^{-/-}$vs T1D or T1D *Nrf2*$^{-/-}$, ♯p<0.05, ♯♯p<0.01 Hypo vs. No Hypo.

Protein (pg/mg)	**Control**	**Control + Hypo**	**T1D**	**T1D + Hypo**		**Nrf2$^{-/-}$**	**Nrf2$^{-/-}$ + Hypo**	**T1D Nrf2$^{-/-}$**	**T1D Nrf2$^{-/-}$ + Hypo**
IFNg	1.26±0.19	1.01±0.19	1.21±0.19	1.16±0.29		2.04 ±0.13	1.99±0.24	1.97±0.21	1.73±0.30
KC/GRO	57.60±7.42	68.86±10.73	65.15±9.51	59.57±7.03		99.49±7.24	95.35±12.47	102.7±12.12	116.7±21.47
IL-1b	1.63±0.14	1.72±0.26	2.68±0.25**	3.15±0.43**♯		2.96±0.49	2.62±0.39	6.95±0.80*	9.40±1.23*♯
IL-2	5.20±1.10	6.19±0.93	6.80±1.27*	10.72±1.23*♯		6.47±0.76	7.10±1.10	10.91±1.15**	10.88±0.86**♯
TNFa	1.87±0.45	1.39±0.73	4.28±0.44*	7.37±0.61**♯		4.97±0.86	7.34±1.40	10.41±2.28*	10.75±1.98*♯
IL-4	1.56±0.26	1.32±0.26	3.05±0.52**	4.35±0.34**♯		2.16±0.32	3.28±0.53	4.96±0.58**	5.34±0.57**♯
IL-6	85.54±13.29	106.4±16.11	146.8±17.30**	178.3±9.32**♯		99.48±11.59	140.4±3.99♯	145.4±8.58**	149.6±6.88**♯

IL-12p70	20.20±1.31	25.84±1.15$^{\#}$	24.83±1.71	27.65±02.09*$^{\#}$		20.00±3.25	27.63±2.83$^{\#\#}$	22.02±3.84*	35.23±4.27*$^{\#\#}$
IL-5	5.39±0.68	7.17±0.41$^{\#}$	5.11±0.69	8.48±0.74*$^{\#\#}$		5.48±0.66	6.78±0.31$^{\#}$	8.45±0.59**	7.58±0.22**$^{\#}$

.

Figure 1

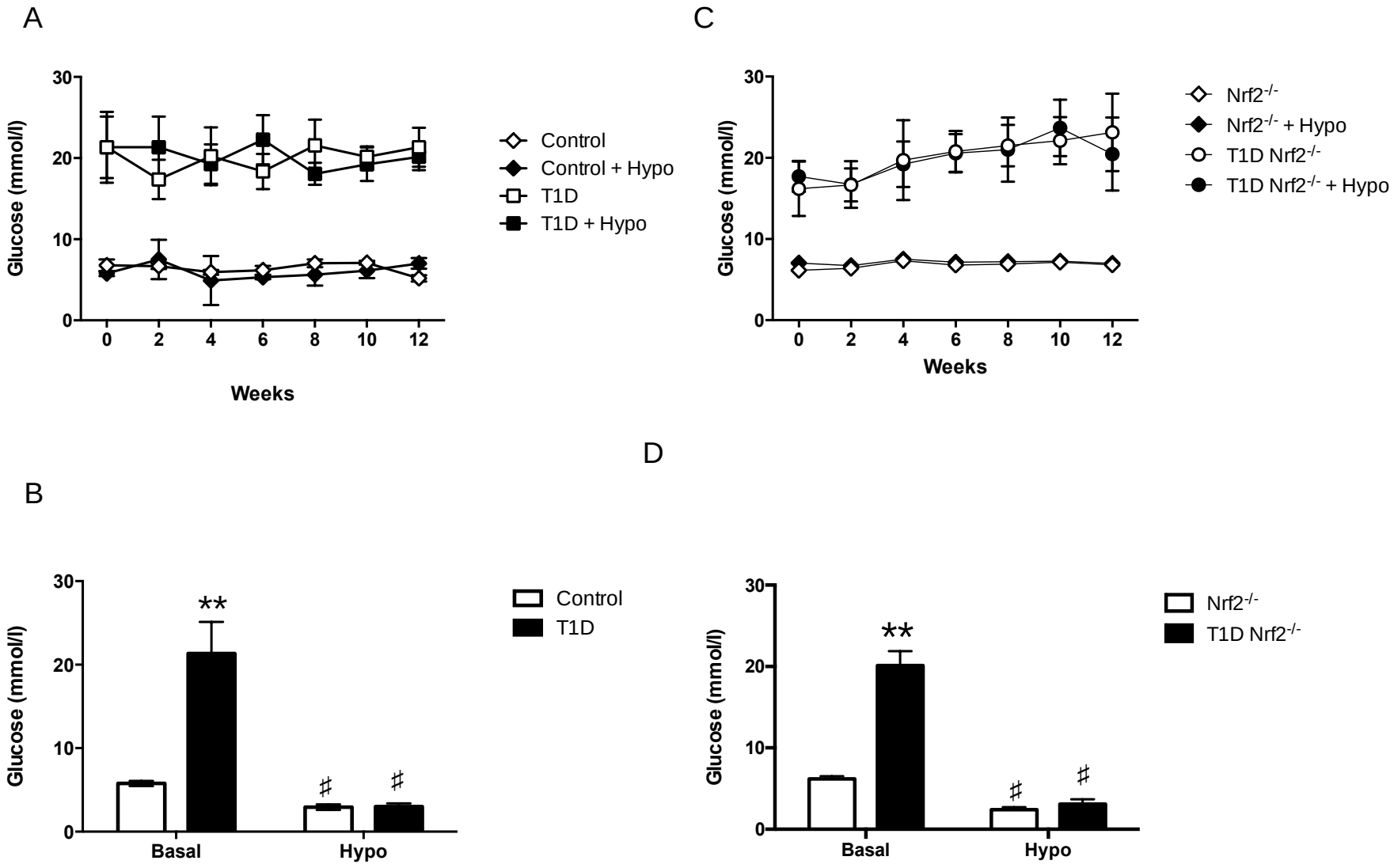

A
Glucose (mmol/l)
30
20
10
0
0
2
4
6
8
10
12
Weeks
Control
Control + Hypo
T1D
T1D + Hypo
C
Glucose (mmol/l)
Weeks
Nrf2-/-
Nrf2-/- + Hypo
T1D Nrf2-/-
T1D Nrf2-/- + Hypo
B
Glucose (mmol/l)
**
#
#
Basal
Hypo
Control
T1D
D
Glucose (mmol/l)
**
#
#
Basal
Hypo
Nrf2-/-
T1D Nrf2-/-

Figure 2

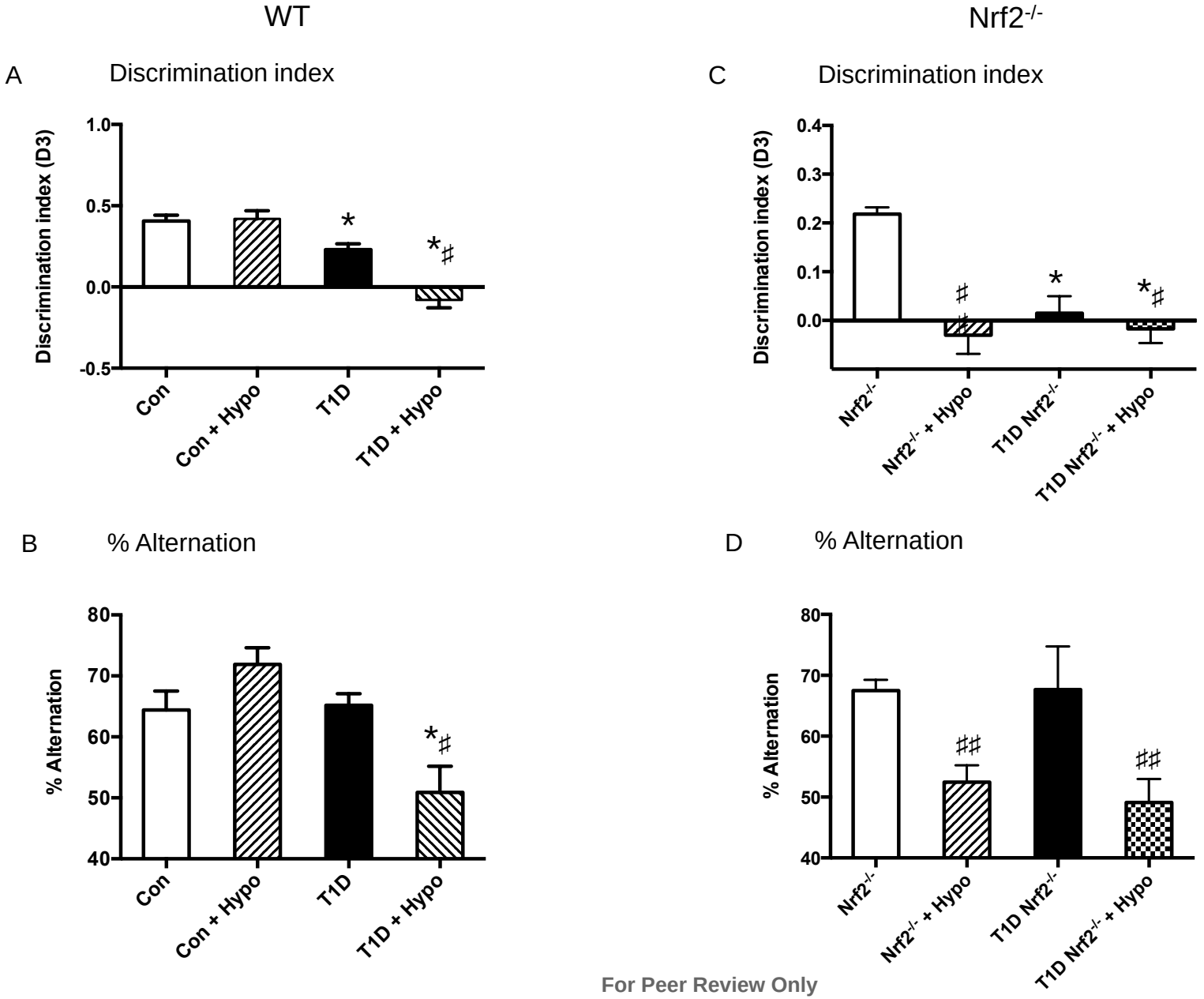
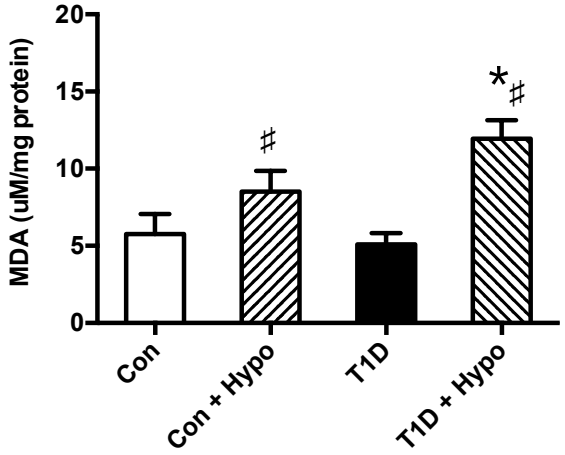

WT
Nrf2-/-
A
Discrimination index
Discrimination index (D3)
1.0
0.5
0.0
-0.5
*
*#
Con
Con + Hypo
T1D
T1D + Hypo
C
Discrimination index
Discrimination index (D3)
0.4
0.3
0.2
0.1
0.0
#
#
*
*#
Nrf2-/-
Nrf2-/- + Hypo
T1D Nrf2-/-
T1D Nrf2-/- + Hypo
B
% Alternation
% Alternation
80
70
60
50
40
*#
Con
Con + Hypo
T1D
T1D + Hypo
D
% Alternation
% Alternation
80
70
60
50
40
##
##
Nrf2-/-
Nrf2-/- + Hypo
T1D Nrf2-/-
T1D Nrf2-/- + Hypo

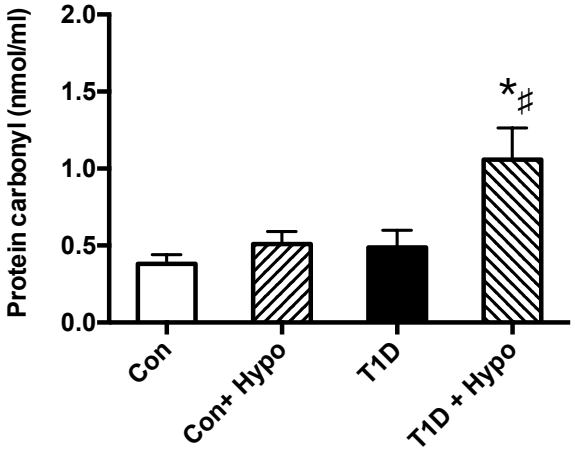
Figure 3

WT

A Lipid Peroxidation

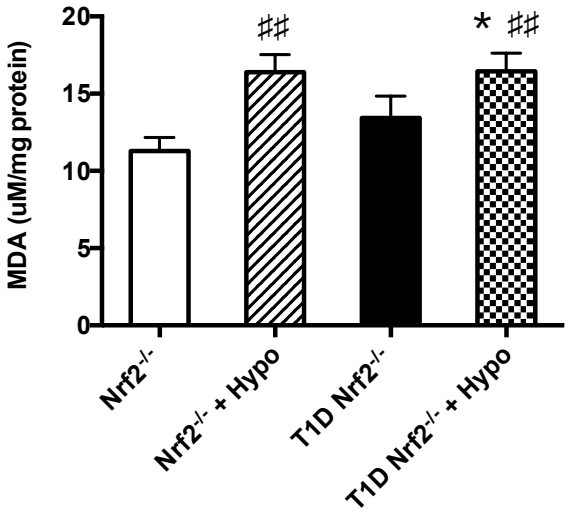


B Protein Carbonylation

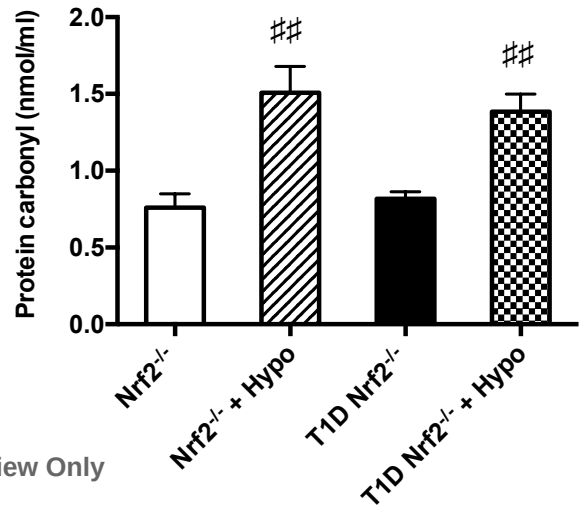


Nrf2$^{-/-}$

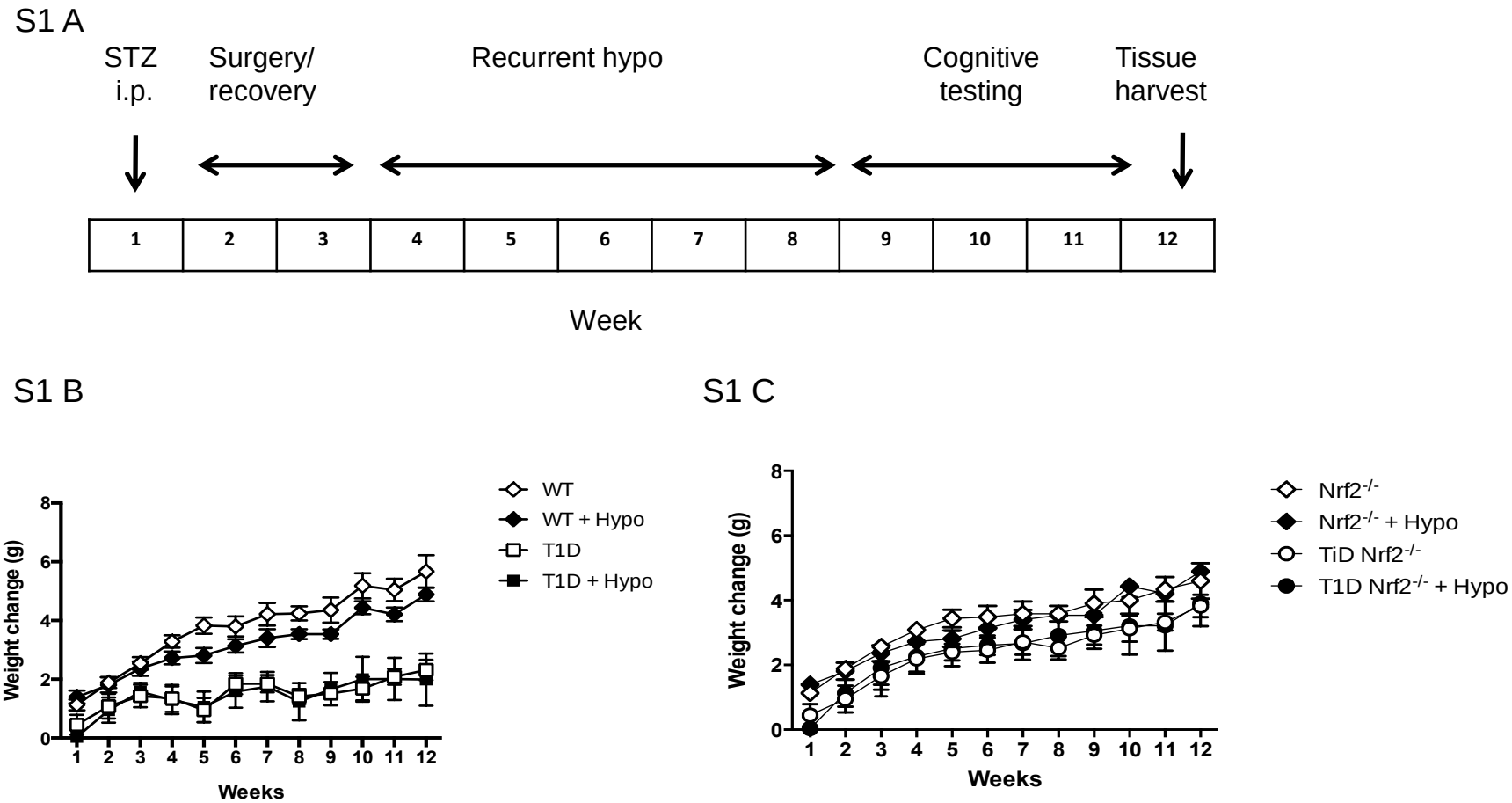
C Lipid Peroxidation



D Protein Carbonylation



Supplementary Figure 1. Time line and weight profile of control and T1D WT and *Nrf2*$^{-/-}$ mice exposed to recurrent hypoglycemia. A. Timeline for induction of diabetes, surgery, recurrent hypoglycemia, behavioral testing and biochemical analysis. B. Body weight was comparable between groups and unaltered by either genotype or RH. Results represent mean values ± SEM.



Supplementary Table 1. Details of Applied Biosystems TaqMan® gene expression assays used for real-time PCR analysis.

Gene	Applied Biosystems assay ID
Hmox-1	Mm00516005_m1
Nrf2	Mm00477784_m1
Nqo1	Mm01253561_m1
IL-6	Mm00446190_m1
Tnfa	Mm00443260_g1
IL-1b	Mm00434228_m1
Nos2	Mm00440502_m1
Txnrd1	Mm00443675_m1
Srxn1	Mm00769566_m1
Txn1	Mm00726847_s1
Gsta1	Hs00275575_m1

Supplementary Table 2. Recurrent hypoglycemia and T1D enhance the expression of inflammatory genes within the hippocampus in WT and *Nrf2*$^{-/-}$ mice. mRNA was extracted and processed for real-time PCR to evaluate changes in gene expression Interleukin 6 (*IL-6*), Tumor necrosis factor alpha (Tnf-α), Interleukin -1beta (IL–1β) and Inducible nitric oxide synthase (*Nos2*). Con, Con + Hypo, T1D and T1D + Hypo all n=8 per group. *Nrf2*$^{-/-}$, *Nrf2*$^{-/-}$+ Hypo, T1D *Nrf2*$^{-/-}$ and T1D *Nrf2*$^{-/-}$+ Hypo all n=7-8 per group. Results represent mean values ± SEM. Data were analyzed within genotype by 2-way ANOVA with T1D and Hypo as between subject factors followed by Tukey's post hoc test * p<0.05, test *p<0.05, **p<0.01 Control or *Nrf2*$^{-/-}$vs T1D or T1D *Nrf2*$^{-/-}$, ♯p<0.05, ♯♯p<0.01 Hypo vs. No Hypo.

Gene	Control	Control + Hypo	T1D	T1D + Hypo	Nrf2$^{-/-}$	Nrf2$^{-/-}$ + Hypo	T1D Nrf2$^{-/-}$	T1D Nrf2$^{-/-}$ + Hypo
IL-6	1.00±0.20	1.13±0.20	1.98±0.37	2.94±0.43	0.88±0.09	0.51±0.03	1.27±0.17**	1.66±0.03**
Tnfa	1.00±0.18	1.23±0.32	1.87±0.24	3.02±0.48	0.79±0.04	0.87±0.07	1.45±0.15**	1.50±0.20**
IL-1b	1.00±0.31	0.70±0.08	0.95±0.13	0.98±0.14	0.17±0.02	0.15±0.02	0.24±0.02**	0.30±0.06**
Nos2	1.00±0.28	1.57±0.33	1.47±0.24	2.79±0.46	0.17±0.02	0.15±0.02	0.245±0.02**	0.30±0.06**